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(54) **CNP analog peptides and their use.**

(57) Novel peptides represented by the general formula:

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(A)-(B)-(C)-Gly-(D)-(E)-(F)-Asp-Arg-Ile-Gly-(G)-(H)-
     :.............:
Ser-Gly-Leu-Gly-(B)-(I)
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and physiologically acceptable acid addition salts thereof;
where
(A) represents H-, H-Gly, H-Lys-Gly, H-Ser-Lys-Gly, H-Leu-Ser-Lys-Gly, H-Gly-Leu-Ser-Lys-Gly, H-ser, H-Ser-Ser, H-Arg-Ser-Ser, H-Arg-Arg-Ser-Ser, H-Leu-Arg-Arg-Ser-Ser, H-Ser-Leu-Arg-Arg-Ser-Ser;
(B) represents H-Cys or Pmp;
(C) represents Phe-, pCl-Phe, pF-Phe, pNO_2-Phe or Cha;
(D) represents Ile, Val, Aib, tLeu, Gly or Leu;
(E) represents Lys or Arg;
(F) represents Ile, Leu or Met;
(G) represents Ser or Ala;

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(H) represents Met or Gln;
(I) represents -OH, -Asn-OH, -Asn-Ser-OH, -Asn-Ser-Phe-OH, -Asn-Ser-Phe-Arg-OH or -Asn-Ser-Phe-Arg-Tyr-OH; and the symbol "....." represents a disulfide bond;

provided that 1) α-hANP, 2) α-hANP (7 - 28) and 3) CNP-22 are excluded from the scope of that general formula.

Also disclosed are agents for suppressing the growth of vascular smooth muscle cells that contains those peptides as effective ingredients.

This invention relates to the synthesis and use of peptides capable of suppressing the growth of vascular smooth muscle cells. More particularly, this invention relates to the synthesis of novel derivatives of C-type natriuretic peptide (hereunder abbreviated as "CNP"), the novel physiological actions of CNP and its derivatives, and to vascular smooth muscle cell growth suppressing agent that contains one of those peptides as an effective ingredient. The term "CNP analog derivatives" as used herein means the compound recited in appended claim 1, CNP-22, human CNP-53, porcine CNP-53, frog CNP and chick CNP.

Many peptides having natriuretic and hypotensive actions have recently been found in the hearts and brains of various animals. These peptides are collectively referred to as "natriuretic peptides" or "NPs". Many NPs having different chain lengths or similar primary amino acid sequences have heretofore been isolated and identified from living bodies and it has now become clear that all of those NPs are biosynthesized from three different NP precursor proteins (prepro ANP, prepro BNP and prepro CNP).

Therefore, NPs known today can be classified as the following three types in accordance with the route of their biosynthesis: A-type NP (A-type natriuretic peptide or ANP); B-type NP (B-type natriuretic peptide or BNP); and C-type NP (C-type natriuretic peptide or CNP).

Among these NPs, ANP and BNP were isolated from the atrium and the brain, respectively, so ANP has initially been called an atrial natriuretic peptide and BNP, a brain natriuretic peptide (Natsuo, H and Nakazato, H., Endocrinol. Metab. Clin. North Amer., 16, 43, 1987; Sudoh, T et al., Nature, 332, 78, 1988). However, later studies have revealed that ANP is produced not only in the atrium but also in the brain and that similarly, BNP is produced not only in the brain but also in the heart (Ueda, S. et al., Biochem. Biophys. Res. Commun., 149, 1055, 1987; Aburaya, M. et al., Biochem. Biophys. Res. Commun., 165, 872, 1989). It was also verified that both ANP and BNP, when administered in vivo, exhibited comparable and noticeable levels of natriuretic and hypotensive actions. On the basis of those findings, both ANP and BNP are presently considered to work not only as hormones to be secreted from the heart into blood but also as nerve transmitting factors, thereby playing an important role in maintaining the homeostatic balance of body fluid volume and blood pressure.

CNP is a group of peptides that are assignable to a third class of NPs following ANP and BNP and those peptides were isolated very recently, followed by the unravelling of their structures and the mechanism of their biosynthesis.

The first discovered CNPs were CNP-22 composed of 22 amino acid residues and CNP-53 having 31 amino acid residues attached to the N-terminus of CNP-22, and those peptides were both isolated from porcine brain and their structures were unravelled. It was also determined that CNP-22 and CNP-53 were present in nearly equal amounts in the porcine brain (Sudoh, T. et al., Biochem. Biophys. Res. Commun., 168, 863, 1990; Minamino, N. et al., Biochem. Biophys. Res. Commun., 170, 973, 1990). At a later time, porcine CNP genes and cDNA corresponding to CNP-22 and CNP-53 were isolated and their analysis has shown that both CNP-22 and CNP-53 are produced from a common precursor protein (prepro CNP). It was also found that this prepro CNP was clearly different from ANP and BNP precursor proteins (prepro ANP and prepro BNP) (Tawaragi, Y. et al., Biochem. Biophys. Res. Commun., 172, 627, 1990).

The isolation of the porcine CNP gene was followed by the isolation of rat CNP cDNA and human CNP gene and the structures of rat and human CNP precursor proteins have been unravelled. As a result, it has been found that CNP-22 has the same primary amino acid sequence in the three animal species, pig, human and rat, that CNP-53 has the same primary amino acid sequence in pig and rat but has different sequences in human and pig in that amino acid substitution occurs in two positions, and that unlike ANP and BNP, CNP is not produced in the heart but produced specifically in the brain (Kojima, M. et al., FEBS letter, 176, 209, 1990; Tawaragi, Y. et al., Biochem. Biophys. Res. Commun., 175, 645, 1991). As of today, a peptide assignable to CNP has also been isolated and identified from frogs and chicks (Japanese Patent Application Nos. 238294/1990 and 238293/1990).

Thus, it has been verified that CNP occurs not only in mammals but also in birds and amphibians. However, much is left unclear about the physiological role of CNP as NP.

The primary amino acid sequence of CNP is similar to those of ANP and BNP and, when administered in vivo, CNP exhibits natriuretic and hypotensive actions. Therefore, CNP has been held assignable to the NP family. However, compared to ANP and BNP, CNP is considerably weak in natriuretic and hypotensive actions (1/50 - 1/100) and, further, unlike ANP and BNP, the tissue of CNP production is limited to the brain; thus, CNP stands in a peculiar position in the NP family and it has been speculated that CNP will play other physiological roles in addition to that of maintaining the homeostatic balance of body fluid volume and blood pressure.

The studies conducted so far have shown that the mechanism by which NP exhibits a hypotensive action will probably be as follows: NP binds to an NP receptor present on the surface of a vascular smooth

muscle cell, thereby increasing the level of intracellular cGMP (cyclic guanosine monophosphate), which works as an intracellular second messenger of NP to eventually cause the relaxation of blood vessels. As a matter of fact, it has been verified that the level of intracellular cGMP rises when NP is allowed to act on a sample of blood vessel or cultured vascular smooth muscle cells (VSMC).

However, the present inventors recently found that when CNP was allowed to act on VSMC, it unexpectedly increased the level of intracellular cGMP in VSMC several times as much as in the case where ANP or BNP was used (Furuya, M. et al., Biochem. Biophys. Res. Commun., 170, 201, 1990). This suggests that cGMP induced by CNP not only works as a second messenger in vascular relaxation but also has a capacity for functioning as a mediator in the development of other physiological actions. In this regard, Garg et al. have shown that vascular relaxants such as nitroprruside and S-nitroso-N-acetyl penillamine suppress cell growth in a rat VSMC line and that 8-bromo cGMP exhibits a similar action in the same cell line. Garg et al. have concluded that this growth suppressing action is caused by cGMP induced by nitric oxide (NO) radicals (Garg, U.C. et al., J. Clin. Invest., 83, 1774, 1989).

Kariya et al. have reported that ANP enhances the production of intracellular cGMP in cultured vascular smooth muscle cells derived from rabbit aortas, thereby suppressing the growth of those cells (Kariya, K. et al., Atherosclerosis, 80, 143, 1989).

All the reports mentioned above suggest strongly that cGMP works as a mediator in suppressing the growth of cultured vascular smooth muscle cells and they also suggest the possibility that cGMP induced by CNP also suppresses the growth of cultured vascular smooth muscle cells. However, it is not known today whether CNP suppresses the growth of cultured vascular smooth muscle cells.

On the other hand, it is known that all NPs have a cyclic structure composed of 17 amino acid residues that are formed on the basis of intramolecular S-S bonds. Thus, when the structure of NP is divided into three domains including the common cyclic structure (i.e., an exocyclic N-terminal domain, an endocyclic domain, and an exocyclic C-terminal domain), it can be seen that the structure of CNP differs from those of ANP and BNP in the following points (see Fig. 1). First, the primary amino acid sequence of CNP completely differs from that of ANP or BNP in terms of the exocyclic N-terminal domain whereas the endocyclic domain of CNP differs from that of ANP in terms of 5 of the 17 amino acid residues and differs from that of BNP in terms of 4 of the 17 amino acid residues. The structure of the exocyclic C-terminal domain of CNP differs greatly from that of ANP or BNP since it does not have the "tail" structure which occurs in ANP or BNP (in the case of ANP and BNP, 5 or 6 amino acids are attached to the C-terminal side of the cyclic structure of ANP or BNP, respectively, and this structure is named a "tail" structure for the sake of convenience). Obviously, these structural differences between CNP and ANP or BNP contribute to the development of the aforementioned characteristic physiological actions of CNP. However, it has not yet been known as to which domain structure of CNP and which primary amino acid sequence are directly involved in the strong cGMP producing activity of CNP.

A first object, therefore, of the present invention determines as to whether peptides assignable to CNP isolated from the nature will suppress the growth of cultured vascular smooth muscle cells.

A second object of the present invention is to unravel the minimum activity structure of CNP that contributes to the development of its strong cGMP producing activity, as well as the primary sequence of the essential amino acids of CNP. On the basis of the thus obtained findings, the present invention aims at constructing novel CNP derivatives that exhibit a stronger cGMP producing activity than naturally derived CNP.

A third object of the present invention is to find a method of using naturally derived CNP and its derivatives as pharmaceutical drugs.

To begin with, in order to achieve the first object of the invention, the present inventors checked to see whether or not CNP would suppress the DNA synthesis of cultured rat vascular smooth muscle cells stimulated with sera or PDGF (platelet derived growth factor).

Then, in order to achieve the second object of the invention, the present inventors constructed those derivatives of α-hANP (in humans, this peptide is primarily secreted from the atrium into blood) and CNP-22 in which part of the amino acid sequences were interchanged with each other, as well as a CNP-22 derivative from which the exocyclic N-terminal domain was deleted, and established which domain or primary amino acid sequence of CNP would contribute to the development of the strong cGMP producing activity which was characteristic of CNP. At the same time, the present inventors unravelled the minimum activity structure which was necessary for the development of the aforementioned activity of CNP. The present inventors then replaced some amino acid residues in those CNP derivatives by unusual amino acid (non-native type) residues so as to construct CNP derivatives that had a stronger cGMP producing activity and DNA synthesis inhibiting activity than CNP.

In order to achieve the third object of the invention, the present inventors entirely reviewed the

previously known physiological actions of CNP including those disclosed by the present invention, as well as the observations heretofore obtained on the basis of the analyses of various diseased tissues and thereby present a specific method of using CNP and novel CNP derivatives as pharmaceutical drugs.

Fig. 1 is a diagram showing the primary structures of representative natriuretic peptides that belong to the respective classes of A-type NP, B-type NP and C-type NP;

Fig. 2 is a graph showing the DNA synthesis inhibiting action of α-hANP and CNP-22;

Fig. 3 is a graph showing the DNA synthesis inhibiting action of α-hANP, CNP-22 and hCNP-53; and

Fig. 4 is a graph showing the correlation between the cGMP producing activity and DNA synthesis inhibiting activity of CNP analog peptides.

In accordance with the method described in Example 1 under 1-2 (see below), the present inventors made an investigation to see whether CNP-22 and human CNP-53 (hCNP-53) would suppress the cell growth of rat VSMC. It was found that as shown in Figs. 2, 3 and 4, both CNP-22 and hCNP-53 would suppress the DNA synthesis of rat VSMC in a dose-dependent manner and with a comparable intensity. It was also found that the intensity of their action was 10 times as great as α-hANP. Further, it was found that as shown in Table 1 below, CNP-22 suppressed the increase in the cell count of VSMC as stimulated with serum.

Table 1 Suppression by CNP-22 and α-hANP of the growth of rat vascular smooth muscle cells stimulated with 1% serum

Compound	Dose	Cell count ($\times 10^3$ cells/well)	[% of control]
Physiological saline		20.6 ± 0.2	[100]
CNP-22	5×10^{-7} M	15.0 ± 0.3 *	[72.8 ± 1.1]
α-hANP	5×10^{-7} M	17.1 ± 0.2 **	[83.0 ± 1.3]

Data shown as mean ± s.d. (n = 12)

* : $p < 0.01$, with statistically significant difference in Student's t-test.

** : $p < 0.05$, with statistically significant difference in Student's t-test.

On the basis of the data shown above, it was discovered for the first time that CNPs (CNP-22 and hCNP-53) exhibited the ability to suppress the cell growth of rat VSMC. It was also found that a positive correlation existed between the intensity of their ability to suppress cell growth and the concentration of intracellular cGMP.

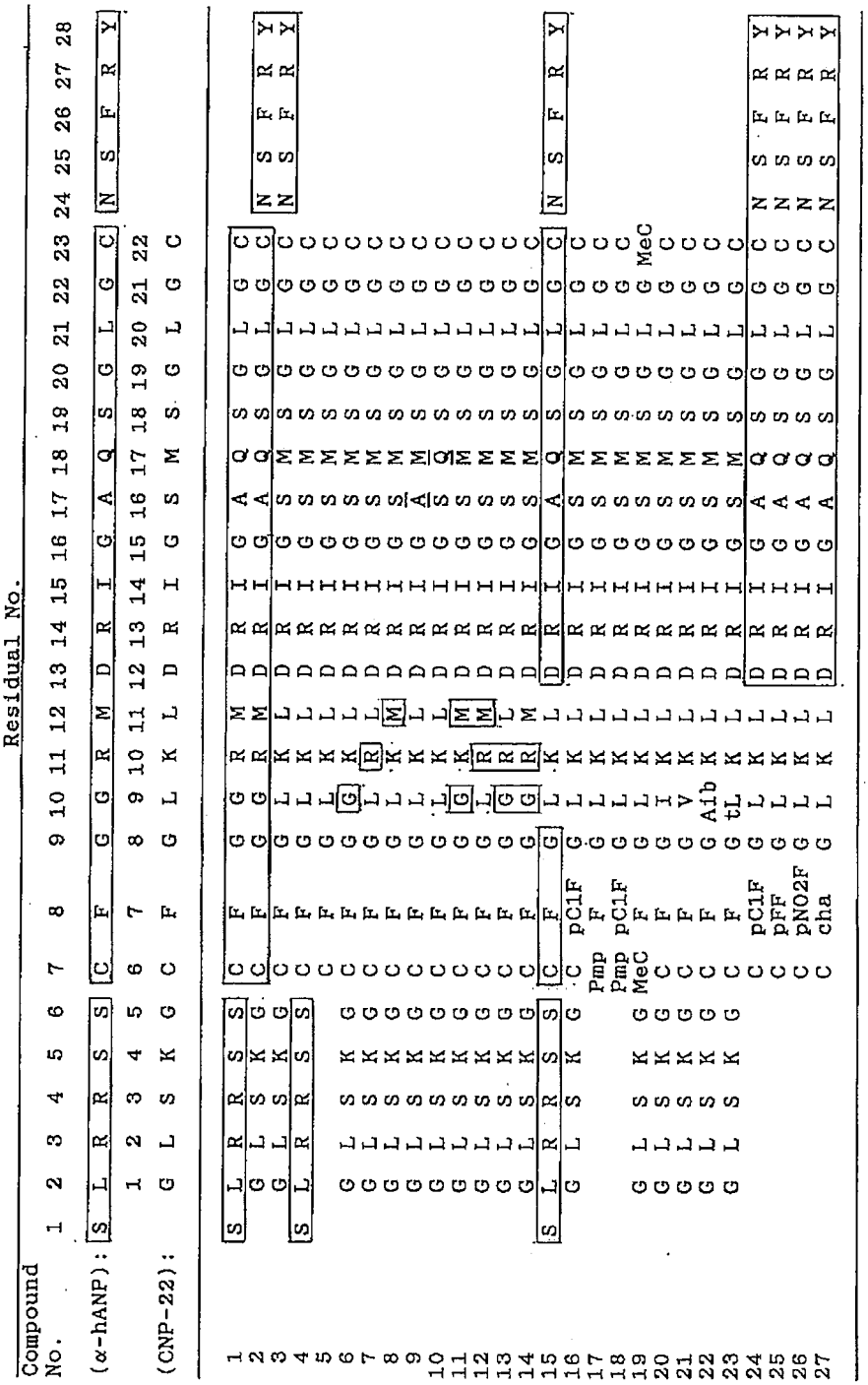
The present inventors then constructed various CNP derivatives in accordance with the guideline to be described below and made an investigation as to which structure or primary amino acid sequence of CNP would be responsible for the strong cGMP producing activity characteristic of CNP.

As already mentioned, structural comparison of CNP-22 with α-hANP as regards three domains (exocyclic N-terminal domain, endocyclic domain, and exocyclic C-terminal domain) shows that CNP-22 differs from α-hANP in the following points (see Fig. 1). First, the primary amino acid sequence of CNP-22 differs entirely from α-hANP in terms of the exocyclic N-terminal domain and as regards the endocyclic domain, CNP-22 differs from α-hANP in five out of the 17 amino acid residues (9-position leucine, 10-

position lysine, 11-position leucine, 16-position serine and 17-position methionine residues in CNP-22, provided that those residues correspond to 10-position glycine, 11-position arginine, 12-position methionine, 17-position alanine and 18-position glutamine residues in α-hANP). Further, CNP-22 does not have the exocyclic C-terminal domain which is present in α-hANP. Therefore, it is obvious that these structural differences are responsible for the differences in physiological action between CNP and ANP (especially the difference in cGMP producing activity against VSMC).

Under these circumstances, the present inventors conducted investigations to determine which domain structure of CNP was responsible for the cGMP producing activity characteristic of CNP. Table 2 below shows the primary structures of all the derivatives synthesized in accordance with the present invention.

Table 2 Amino acid sequences of novel CNP derivatives



Compound No.	Residual No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
(α-hANP):	S	L	R	R	S	S	C	F	G	G	R	M	D	R	I	G	A	Q	S	G	L	G	C	N	S	F	R	Y
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23				
(CNP-22):		G	L	S	K	G	C	F	G	L	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
1	S	L	R	R	S	S	C	F	G	G	R	M	D	R	I	G	A	Q	S	G	L	G	C	N	S	F	R	Y
2		G	L	S	K	G	C	F	G	G	R	M	D	R	I	G	A	Q	S	G	L	G	C	N	S	F	R	Y
3		G	L	S	K	G	C	F	G	L	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
4	S	L	R	R	S	S	C	F	G	L	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
5							C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
6						G	C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
7						G	C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
8						G	C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
9						G	C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
10						G	C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
11						G	C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
12						G	C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
13						G	C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
14						G	C	F	F	G	K	L	D	R	I	G	S	M	S	G	L	G	C	N	S	F	R	Y
15	S	L	R	R	S	S	C	F	G	L	K	L	D	R	I	G	A	Q	S	G	L	G	C	N	S	F	R	Y
16		G	L	S	K	G	C	pC1F	G	L	K	L	D	R	I	G	S	M	S	G	L	G	C					
17							C	F	G	L	K	L	D	R	I	G	S	M	S	G	L	G	C					
18							C	Pmp	G	L	K	L	D	R	I	G	S	M	S	G	L	G	C					
19							C	Pmp	G	L	K	L	D	R	I	G	S	M	S	G	L	G	C	MeC				
20		G	L	S	S	K	C	MeC	G	L	I	K	L	D	R	I	G	S	M	S	G	L	G	C				
21		G	L	S	S	K	C	F	G	V	K	L	D	R	I	G	S	M	S	G	L	G	C					
22		G	L	S	S	K	C	F	G	Aib	K	L	D	R	I	G	S	M	S	G	L	G	C					
23		G	L	S	S	K	C	F	G	tL	K	L	D	R	I	G	S	M	S	G	L	G	C					
24							C	pC1F	G	L	K	L	D	R	I	G	A	Q	S	G	L	G	C	N	S	F	R	Y
25							C	pFF	G	L	K	L	D	R	I	G	A	Q	S	G	L	G	C	N	S	F	R	Y
26							C	pNO2F	G	L	K	L	D	R	I	G	A	Q	S	G	L	G	C	N	S	F	R	Y
27							C	cha	G	L	K	L	D	R	I	G	A	Q	S	G	L	G	C	N	S	F	R	Y

First, the inventors synthesized an α-hANP derivative having the exocyclic C-terminal domain deleted, CNP-22 and α-hANP derivatives having the respective domains interchanged therebetween, and a CNP-22 derivative having the exocyclic N-terminal domain deleted (see Table 2 under 1 - 5), and they investigated the cGMP producing activity of those derivatives.

As shown in Table 3 below, it was found that each of derivatives 3 and 4 which had the endocyclic

domain structure of CNP-22 in their molecule, and derivative 5 which had the exocyclic N-terminal domain of CNP-22 deleted exhibited a strong cGMP producing activity at a comparable level to that exhibited by CNP-22.

Table 3 Physiological activities of CNP analogs

Compound	% Inhibition of ^{3}H-thymidine uptake[1)]	cGMP producing activity (% increase for 1 μm)[2)]
α-hANP	58	100
CNP-22	76	621
1	NT	62
2	38	147
3	NT	667
4	NT	663
5	65	616
6	49	344
7	NT	413
8	NT	497
9	NT	707
10	NT	659
11	NT	71
12	NT	273
13	NT	58
14	17	23
15	72	559
16	NT	688
17	NT	233
18	NT	333
19	NT	38
20	NT	499
21	NT	549
22	NT	160
23	NT	NT
24	NT	719
25	NT	785
26	NT	255
27	NT	523
hCNP-53	71	458
pCNP-53	NT	524

1) Percent inhibition as achieved by adding 0.1 μm of each peptide to a BSMC line stimulated with PDGF (20 ng/mℓ).
2) Specific activity as compared to the activity of 1 μm α-hANP (2000 fmol/400000 cells), with maximum activity being equivalent to the cGMP producing ability of each compound for 1 μm.

On the other hand, derivative 1 having the exocyclic C-terminal domain of α-hANP deleted and derivative 2 in which the exocyclic N-terminal domain of α-hANP was replaced by the exocyclic N-terminal domain of CNP-22 were found to exhibit only a weak cGMP producing activity at a level comparable to α-hANP.

On the basis of the data described above, it was concluded that the strong cGMP producing activity of CNP was due to the endocyclic domain structure of CNP-22. Further, it was eventually found that derivative 19 which had the intramolecular S-S bonds of CNP-22 cleaved hardly reduced the cGMP producing activity and this fact enabled the present inventors to conclude that cyclic CNP (6 - 23) 5 was the minimum activity structure regarding the cGMP producing activity of CNP.

Subsequently, the present inventors made an investigation as to which of the amino acid residues (or which primary amino acid residue) in the endocyclic domain of CNP was important for the development of the cGMP producing activity of CNP. To this end, using CNP-22 as the skeletal structure, the present inventors constructed one-residue substituted derivatives (see Table 2 under 6 - 10) in which 5 amino acid residues in the endocyclic domain of CNP-22 that were different than α-hANP (i.e., 9-position leucine, 10-position lysine, 11-position leucine, 16-position serine and 17-position methionine residues) were replaced by the corresponding amino acid residues in α-hANP (i.e., 10-position glycine, 11-position arginine, 12-position methionine, 17-position alanine and 18-position glutamine), and their activity for cGMP production was investigated.

As shown in Table 3, both derivatives 9 (16-position substituted derivative of CNP-22) and 10 (17-position substituted derivative) exhibited a strong activity comparable to that of CNP-22. On the other hand, derivatives 6, 7 and 8 (9-, 10- and 11-position substituted derivatives, respectively, of CNP-22) had a lower activity than CNP-22 (but higher than that of α-hANP).

On the basis of these data, it was found that the 9-position leucine, 10-position lysine and 11-position leucine residues of CNP-22 were important for the development of the cGMP producing activity of CNP. However, as is clear from Table 3, none of the one-residue substituted derivatives (6, 7 and 8) achieved satisfactory reduction in activity; therefore, it was anticipated that the residues important to the activity were not single residues but that they would be composed of at least two residues selected from among the 9-position leucine, 10-position lysine and 11-position leucine residues of CNP-22.

Therefore, the present inventors subsequently constructed two-residue substituted derivatives (see Table 2 under 11 - 13) and three-residue substituted derivative (see Table 2 under 14) by combining the 9-position leucine, 10-position lysine and 11-position leucine residues of CNP-22 and investigated their cGMP producing activity.

As shown in Fig. 3, the three-residue substituted derivative 14 caused a marked drop in activity as was expected. Each of the two-residue substituted derivatives caused an even greater drop in activity than the aforementioned one-residue substituted derivatives. The drop in activity caused by derivatives 11 and 13 was particularly marked.

On the basis of those analyses, it was found that Leu-Lys-Leu, namely, the sequence of positions 9 to 11 of the primary amino acid sequence for the endocyclic domain of CNP-22 was important for the development of the cGMP producing activity of CNP. This was also verified by the fact that three-residue substituted derivative 15 in which the amino acid residues in positions 10, 11 and 12 of α-hANP were replaced by leucine, lysine and leucine residues, respectively, exhibited a strong cGMP producing activity at a substantially comparable level to CNP-22. It is difficult to identify which of the three-residues in positions 9 - 11 of CNP-22 is particularly important on the basis of the above-described experimental results but in view of the fact that the one-residue substituted derivative 6 and the two-residue substituted derivatives 11 and 13 were all lower in cGMP producing activity than the other one- and two-residue substituted derivatives, the leucine residue in position 9 of CNP may well be considered to be particularly important. In other words, the difference in cGMP producing ability between ANP and CNP would originate from the difference between the leucine residue in position 9 of CNP-22 and the corresponding glycine

residue in position 10 of α-hANP.

In the next place, combining those findings of the present invention with the observations obtained by the previous studies on the structure-activity correlation of ANP (see, for example, Minamitake, Y. et al., Biochem. Biophys. Res. Commun., 172, 971, 1990), the present inventors attempted to prepare CNP derivatives having a stronger cGMP producing activity and DNA synthesis inhibiting activity than naturally occurring NP (ANP or CNP).

First, noting the leucine residue in position 9 of CNP-22, the present inventors constructed derivatives that had CNP-22 as the skeletal structure and in which the leucine residue in position 9 was replaced by isoleucine, valine, α-aminoisobutyric acid or t-leucine residue (see Table 2 under 20 - 23). Then, the inventors constructed other derivatives that had CNP (6 - 22) 5 as the skeletal structure and in which the cysteine residue in position 6 was replaced by a pentacyclomercaptopropionyl group, the phenylalanine residue in position 7 was replaced by a p-chloro-phenylalanine residue, and the 6- and 7-position residues were respectively replaced by pentacyclomercaptopropionyl and p-chloro-phenylalanine residues (see Table 2 under 16, 17 and 18). Further, the inventors constructed other derivatives that had [Leu10, Lys11, Leu12] α-hANP (7 - 28) as the skeletal structure and in which the phenylalanine residue in position 8 was replaced by p-chloro-phenylalanine, p-fluoro-phenylalanine, p-nitrophenylalanine and cyclohexylalanine residues, respectively (see Table 2 under 24 - 27). The inventors also investigated the cGMP production by those derivatives.

As shown in Table 3, among the derivatives having CNP-22 as the skeletal structure, derivatives 20 and 21 were found to exhibit substantially the same level of activity as CNP-22. As for the derivatives having CNP (6 - 22) as the skeletal structure; derivative 16 was found to exhibit a stronger activity than CNP-22. As regards the derivatives having [Leu10, Lys11, Leu12] α-hANP (7 - 28) as the skeletal structure, derivatives 15, 24, 25 and 27 were found to exhibit 4 - 6 times as high activity as α-hANP. Derivatives 24 and 25 were found to have a particularly high activity, even stronger than CNP-22.

On the basis of these results, it was found that derivatives exhibiting a stronger cGMP producing activity than CNP-22 or α-hANP could be constructed by replacing part of the amino acid residues in CNP-22, CNP (6 - 22) and [Leu10, Lys11, Leu12] α-hANP (7 - 28) with unusual amino acid (non-native type) residues.

We now describe specifically the method of using CNP and its derivatives as pharmaceutical drugs.

Up to date, there have been reported various disease caused by the abnormal growth of vascular smooth muscle cells. For example, the restenosis of the coronary artery occurs in about 30% of the patients on whom percutaneous transluminal coronary angioplasty (PTCS) has been performed successfully and it is known that in almost all cases, the cause is not the formation of thrombi but the abnormal growth of arterial smooth muscle cells. it is also known that restenosis of a similar type occurs in blood vessels in transplanted tissues including an artery bypass. Further, the growth of vascular smooth muscle cells has often been found in the blood vessels of patients suffering from arteriosclerosis. However, no therapeutics have yet been found that are effective against those diseases due to the growth of vascular smooth muscle cells and their development is presently in demand.

In the present invention, the inventors have revealed for the first time that CNP is capable of effectively suppressing the growth of vascular smooth muscle cells. The inventors have also revealed that a positive correlation holds between the intensity of this action and that of cGMP producing activity. Further, the inventors succeeded in constructing derivatives exhibiting a stronger cGMP producing activity than naturally occurring ANP or CNP.

With these facts taken together, CNP and its derivatives that exhibit a strong cGMP producing activity against vascular smooth muscle cells can potentially be used as effective therapeutics or preventives against diseases such as restenosis and arteriosclerosis that are caused by the abnormal growth of vascular smooth muscle cells.

To summarize, the present inventors unravelled the fact that CNP strongly suppresses the abnormal growth of vascular smooth muscle cells and further found that a positive correlation would hold between the intensity of this action and that of cGMP producing activity.

The present inventors then synthesized various novel derivatives of CNP and found that CNP (6 - 22) was the minimum structure required for the cGMP producing activity of CNP against VSMC. The present inventors also succeeded in synthesizing novel CNP derivatives that would exhibit a stronger cGMP producing activity than naturally occurring ANP or CNP. Further, the present inventors found that CNP and its derivatives could be used as effective therapeutics or preventives against diseases such as restenosis and arteriosclerosis that are caused by the abnormal growth of vascular smooth muscle cells. The present invention has been accomplished on the basis of those findings.

In specific examples of the present invention, the description concerns the CNP derivatives listed in

Table 2 but it should be noted that in the light of the findings of the present invention, the construction of derivatives exhibiting a stronger cGMP producing activity against VSMC is also applicable to the other NPs the structures of which have already been identified.

The peptides of the present invention may be converted to acid addition salts with inorganic acids such as hydrochloric acid, sulfuric acid and phosphoric acid, or with organic acids such as formic acid, acetic acid, butyric acid, succinic acid and citric acid.

The peptides of the present invention can be produced by standard methods of chemical synthesis or recombinant DNA techniques (except for derivatives that contain amino acid residues of a non-native type). Review books on methods of chemical synthesis include, for example, "Seikagaku Jikken Koza (A Course in Experimental Biochemistry) I, Tanpakushitsu no Kagaku (Protein Chemistry), IV, Part II, pages 207 - 495" published by Tokyo Kagaku Dojin, "Peputido Gosei no Kiso to Jikken (Fundamentals and Experiments of Peptide Synthesis), by N. Izumiya et al.", published by Maruzen, and "Peputido Kemisutori (Peptide Chemistry), 1984, pp. 229 - 234, pp. 235 - 240 and pp. 241 - 246, ed. by Izumiya" ed. by Izumiya", published by Tanpakuken (Protein Engineering Research Laboratory), and various methods of synthesis are described in detail in those books. An example of the production methods by recombinant DNA techniques is described in "Idenshi Sosa (Gene Manipulation), 1990, Extra Issue of Tanpakushitsu Kakusan Koso (Proteins, Nucleic Acid and Enzymes), pages 2613 - 2619, ed. by M. Takanami and K. Kimura", published by Kyoritsu Shuppan, and the basic procedures of production are described in this reference.

The peptides of the present invention were synthesized in accordance with the method of chemical synthesis described in those references. Namely, amino acids with protective groups were condensed and extended by a method known as the solid-phase method" and, after removing all protective groups with hydrogen fluoride, the desired peptides were produced via a disulfide binding reaction.

The crude peptides obtained by the aforementioned methods are purified by combinations of common methods of purification such as ion-exchange column chromatography, reverse-phase column chromatography, etc.

The pharmaceutical composition of the present invention can be administered either as free forms of the peptides of the present invention or as pharmacologically acceptable acid addition salts thereof.

The peptides of the present invention or their pharmacologically acceptable acid addition salts are desirably mixed with a pharmacologically acceptable carrier, excipient, diluent, etc. that are known per se before they are administered by methods that are commonly used with peptide drugs, namely, by parenteral administration such as intravenous, intramuscular or subcutaneous administration. However, they may be administered perorally as microcapsules in which the peptides of the present invention are incorporated as the active ingredient in liposome, polyamide, etc. and which are rendered resistant to degradation in the digestive tract. Another method of administration that can be adopted is to have the drug absorbed through the mucous membrane such as in the rectum, within the nose or eye or beneath the tongue, so that the drug is administered as a suppository, intranasal spray, eye drop or sublingual tablet.

The dose of the pharmaceutical composition of the present invention may vary with the kind of disease, the age of patient, his body weight, the severity of disease, the route of administration, etc.; typically, it can be administered in a daily dose of 0.01 - 10 mg/body, with the preferred range being from 0.05 to 1 mg/body.

Unless otherwise noted, the amino acids mentioned herein are in L-form and the abbreviations for those amino acids and reagents are listed below.

Cha:	cyclohexylalanin
Asp:	L-asparagine
Asp(OcHex):	β-cyclohexylaspartic acid
Ser:	L-serine
Ser(Bzl):	O-benzyl-L-serine
Gln:	L-glutamine
Gly:	Glycine
Ala:	L-alanine
Cys:	L-cysteine
Cys(4MeBzl):	4-methylbenzyl-L-cysteine
Met:	L-methionine
Ile:	L-isoleucine
Leu:	L-leucine
t-Leu:	L-tertiary leucine
Tyr:	L-tyrosine
Tyr(BrZ):	O-2-bromobenzyloxycarbonyl-L-tyrosine

Phe:	L-phenylalanine
Arg:	L-arginine
Arg(Tos):	G-tosyl-L-arginine
pCℓPhe:	parachloro-L-phenylalanine
Pmp:	pentacyclomercaptopropionic acid
Aib:	aminoisobutyric acid
Lys:	L-lysine
Boc-:	t-butyloxycarbonyl
TFA:	trifluoroacetic acid
NMP:	N-methylpyrrolidone
DMSO:	dimethyl sulfoxide
HOBt:	N-hydroxybenzotriazole
DIEA:	diisopropylethylamine
DCC:	dicyclohexylcarbodiimide

The purity of each final product was assayed by the procedures of thin-layer chromatography, analytical high-performance liquid chromatography and amino acid analysis that are described below.

Thin-layer chromatography

Support: silica gel 60 F-254 (Merck)
Developing solvent:
rf1 n-butanol:acetic acid:pyridine:water = 4:1:1:2
rf2 n-butanol:acetic acid:pyridine:water = 30:20:6:24

Analytical high-performance liquid chromatography

Apparatus: Shimadzu LC-6A system
Column: YMC-Pack A-302 0D5 4.6$^{\phi}$ X 150 mm
Developing solvent: 30-min linear gradient from 18% CH_3CN/0.1% TFA to 60% CH_3CN/0.1% TFA

Amino acid analysis

Apparatus: Hitachi Amino Acid Analyzer Model 835

The following examples are provided for the purpose of further illustrating the present invention but are in no way to be taken as limiting.

Example 1: Measurements of Biological Activities

1-1: Measurement of CGMP producing activity

The above-mentioned activity of compounds synthesized according to the present invention was measured by the methods of Hirata et al. (Biochem. Biophys. Res. Commun., 128, 538, 1985) and Scarborough et al. (J. Biol. Chem., 261, 12960, 1986). The cells used were cultured vascular smooth muscle cells (hereunder abbreviated as VSMC) derived from the aortas of rats. From 10^{-9} to 10^{-6} M of α-hANP or a peptide of interest was incubated together with VSMC and the amount cGMP produced was measured by cGMP radioimmunoassay. Percent maximum reactivity for each peptide, with the value of maximum reactivity for α-hANP being taken as 100%, was used as an indicator of activity.

1-2: Measurement of cell growth suppressing activity

Cell growth suppressing activity was evaluated in accordance with the method of Kariya et al. (Atherosclerosis, 80, 143 - 147, 1990) by measuring the uptake of [^{3}H] thymidine into cells as an indicator of percent DNA synthesis inhibition using the above-identified VSMC. Cells tuned to a stationary phase were incubated at 37°C for 14 h with each sample added in the presence of 1% serum or 20 ng/mℓ of PDGF (platelet derived growth factor). Then, following the addition of 37 kBg/mℓ of [^{3}H] thymidine, the incubation was continued for another 4 h and the radioactivity of [^{3}H] thymidine incorporated into the cells was measured. The values of measurement were such that the uptake of [^{3}H] thymidine for the case where only 1% serum or PDGF was added in the absence of peptide was taken as 100%, with the percent suppression

due to the peptide addition being accordingly calculated. The results of measurements are shown in Figs. 2 and 3 and in Table 3.

As for CNP-22, VSMC were cultured for 4 days in the presence of 1% serum with CNP-22 or α-hANP being added, and the number of cells was counted with a hemocytometer. The results are shown in Table 1.

Example 2: Synthesis of CNP Derivatives

The peptides of the present invention were all prepared by the solid-phase method with a peptide synthesizer Model 431 of Applied Biosystems, Inc. As representative examples, the synthesis of compounds listed in Table 2 under 2 and 19 is shown below.

2-1: Compound No. 2

Synthesis of H-Gly-Leu-Ser-Lys-Gly-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Glu-Leu-Glu-Cys-Asn-Ser-Phe-Arg-Tyr-OH (of disulfide type)

Starting with 0.7 g (0.5 mmol) of Boc-Tyr(Br-Z)-O-CH2-PAM resin, removal of Boc with 60% TFA, neutralization with DIEA, and condensation of protected amino acids with DCC/HOBt were repeated sequentially to obtain ca. 2.1 g of a protected peptide resin. The resin was treated with HF (17 mℓ) at -2°C for 60 min in the presence of para-cresol (3 mℓ). The free peptide was extracted with 50 mℓ of TFA and thereafter concentrated, followed by addition of ether to obtain 800 mℓ of a crude peptide. This peptide was dissolved in 32 g of urea-saturated water and added dropwise, with stirring, to urea-saturated water (288 mℓ, pH 7.4) containing potassium ferricyanide (147 mg, 44.8 μmol). After the end of the addition, the reaction solution was adjusted to pH 5 with acetic acid and loaded on a linked column of AG3-X4A (10 mℓ, Cℓ-form) and HP-20 (150 mℓ that were equilibrated with 1 N AcOH. After washing with 1 N AcOH (500 mℓ), the peptide adsorbed on HP-20 was eluted with 80% CH_3CN/1 N AcOH. The fractions containing the desired peptide were concentrated and freeze-dried to obtain a crude cyclic peptide (750 mg).

Subsequently, the crude peptide was loaded on an ion-exchange column (CM-2SW, 2$^\phi$ x 15 cm) equilibrated with water and the peptide was eluted by a 60-min linear gradient from water to 0.5 M NH_4OAc (pH 7.2). The principal fractions were collected, loaded on a reverse-phase C18 column (YMC-Pack D-ODS, 2$^\phi$ x 25 cm) initialized with 0.1% TFA, and thereafter subjected to a 60-min linear gradient from 30% CH_3CN/ 0.1% TFA to 60% CH_3CN/0.1% TFA for elution at 10 mℓ/min. Fractions having a purity of at least 97% were collected and freeze-dried to obtain 150 mg of the end compound (2). All other derivatives excepting compound No. 19 were prepared in the manner described above.

2-2: Compound No. 19

Synthesis of H-Gly-Leu-Ser-Lys-Gly-Cys(Me)-Phe-Gly-Leu-Lys-Leu-Asp-Arg-Ile-Gly-Ser-Met-Ser-Gly-Leu-Gly-Cys(Me)-OH

Dithiothreitol (10 mℓ) was added to an aqueous solution (5 mℓ) of CNP (6.0 mg) and the resulting solution was adjusted to pH 8.5 with 10% aqueous ammonia, followed by standing at room temperature for 30 min. The reaction solution was loaded on a reverse-phase C18 column (YMC-Pack D-ODS, 2$^\phi$ x 25 cm) and the desired peptide was isolated by CH_3CN gradient elution in accordance with the procedure described in Example 1 under 1-1, and the isolated peptide was freeze-dried to obtain 5.7 mg (2.6 μmol) of reduced CNP. It was then dissolved in water (3 mℓ) and an acetonitrile solution (0.5 mℓ) containing 1.6 mg (7.3 μmol) of methyl 4-nitrobenzenesulfonate was added to the resulting aqueous solution and the mixture was left to stand at room temperature for 2 h. After verifying the loss of the starting material by HPLC, the peptide was separated on a reverse-phase C18 column to obtain 4.5 mg of the end compound (19).

As for the physiological activities of CNP, it was for the first time found by the present inventors that CNP had a strong cell growth suppressing activity with respect to vascular smooth muscle cells.

This action was found to be 10 times as strong as that of α-hANP. It was also found that a positive correlation holds between the intensity of that action and the concentration of intracellular cGMP.

Then, as for the correlation between the structure and activity of CNP, the present inventors found that the minimum activity structure for the cGMP producing activity of CNP was cyclic CNP (6 - 23) 5. It was also found that Leu-Lys-Leu, or the sequence of positions from 9 to 11 of CNP-22 as the primary amino

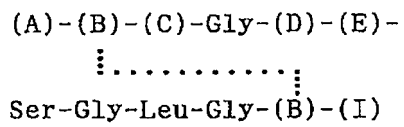
acid sequence of the endocyclic domain of CNP, was important for the development of the cGMP producing activity of CNP.

Further, as regards the synthesis of novel CNP derivatives, the present inventors found that CNP derivatives exhibiting a stronger cGMP producing activity than CNP-22 or α-hANP could be constructed by replacing part of the amino acid residues in CNP-22, CNP (6 - 22) and [Leu10, Lys11, Leu12] α-hANP (7 - 28) with unusual amino acid (non-native type) residues.

On the basis of these findings, CNP and its derivatives that exhibit a strong cGMP producing activity and cell growth suppressing activity against vascular smooth muscle cells are anticipated to have utility as very effective therapeutics or preventives against diseases such as restenosis and arteriosclerosis that are caused by the abnormal growth of vascular smooth muscle cells. In this connection, it should be noted that among the derivatives synthesized in accordance with the present invention that contain unusual amino acid (non-native type) residues would probably exhibit resistance to proteases in the living body (in blood and on the surface of cells) upon administration in vivo. Therefore, those derivatives, even if they have a lower cGMP producing activity than CNP-22 or α-hANP, would be characterized by a longer blood half-life than CNP analogs free from unusual amino acids, and, from this viewpoint, too, those derivatives are anticipated to have industrial utility.

Claims

1. A novel peptide represented by the general formula:

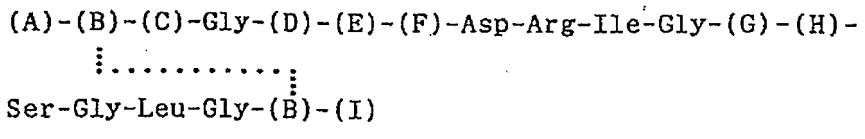


and a physiologically acceptable acid addition salt thereof;
where
A) represents H-, H-Gly, H-Lys-Gly, H-Ser-Lys-Gly, H-Leu-Ser-Lys-Gly, H-Gly-Leu-Ser-Lys-Gly, H-Ser, H-Ser-Ser, H-Arg-Ser-Ser, H-Arg-Arg-Ser-Ser, H-Leu-Arg-Arg-Ser-Ser, H-Ser-Leu-Arg-Arg-Ser-Ser;
(B) represents H-Cys or Pmp;
(C) represents Phe-, pCl-Phe, pF-Phe, pNO_2-Phe or Cha;
(D) represents Ile, Val, Aib, tLeu, Gly or Leu;
(E) represents Lys or Arg;
(F) represents Ile, Leu or Met;
(G) represents Ser or Ala;
(H) represents Met or Gln;
(I) represents -OH, -Asn-OH, -Asn-Ser-OH, -Asn-Ser-Phe-OH, -Asn-Ser-Phe-Arg-OH or -Asn-Ser-Phe-Arg-Tyr-OH; and
the symbol "....." represents a disulfide bond; provided that 1) α-hANP, 2) α-hANP (7 - 28) and 3) CNP-22 are excluded from the scope of that general formula.

2. An agent for suppressing the growth of vascular smooth muscle cells that contains at least one of the CNP analog derivatives as an effective ingredient.

Claims for the following Contracting States : ES, GR

1. A process for preparing a novel peptide represented by the general formula:



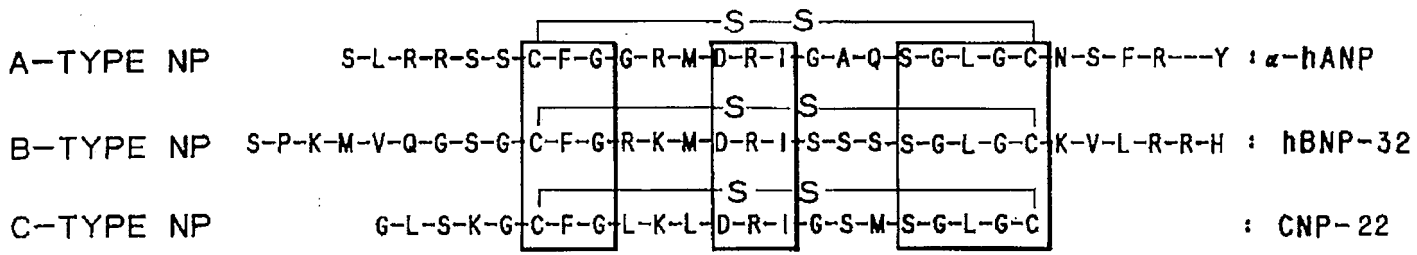
and a physiologically acceptable acid addition salt thereof;
where

(A) represents H-, H-Gly, H-Lys-Gly, H-Ser-Lys-Gly, H-Leu-Ser-Lys-Gly, H-Gly-Leu-Ser-Lys-Gly, H-Ser, H-Ser-Ser, H-Arg-Ser-Ser, H-Arg-Arg-Ser-Ser, H-Leu-Arg-Arg-Ser-Ser, H-Ser-Leu-Arg-Arg-Ser-Ser;
(B) represents H-Cys or Pmp;
(C) represents Phe-, pCl-Phe, pF-Phe, pNO_2-Phe or Cha;
(D) represents Ile, Val, Aib, tLeu, Gly or Leu;
(E) represents Lys or Arg;
(F) represents Ile, Leu or Met;
(G) represents Ser or Ala;
(H) represents Met or Gln;
(I) represents -OH, -Asn-OH, -Asn-Ser-OH, -Asn-Ser-Phe-OH, -Asn-Ser-Phe-Arg-OH or -Asn-Ser-Phe-Arg-Tyr-OH; and the symbol "....." represents a disulfide bond; provided that 1) α-hANP, 2) α-hANP (7 - 28) and 3) CNP-22 are excluded from the scope of that general formula by a chemical "solid-phase" method or by recombinant DNA techniques.

2. Use of a novel peptide obtainable by a process according to claim 1 for the manufacture of an agent for suppressing the growth of vascular smooth muscle cells that contains at least one of the CNP analog derivatives as an effective ingredient.

Fig. 1

PRIMARY STRUCTURES OF NATRIURETIC PEPTIDE FAMILIES



SHOWN ARE THE PRIMARY STRUCTURES OF PEPTIDES REPRESENTATIVE OF THE RESPECTIVE TYPES. WITH THE AMINO ACID SEQUENCES COMMON TO THE ENDOCYCLIC DOMAIN BEING WITHIN BOX.

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Fig. 2

INHIBITION BY α-hANP, CNP-22 AND 8-br-cGMP OF DNA SYNTHESIS BY CULTURED RAT VASCULAR SMOOTH MUSCLE CELLS IN RESPONSE TO STIMULATION WITH 1% SERUM

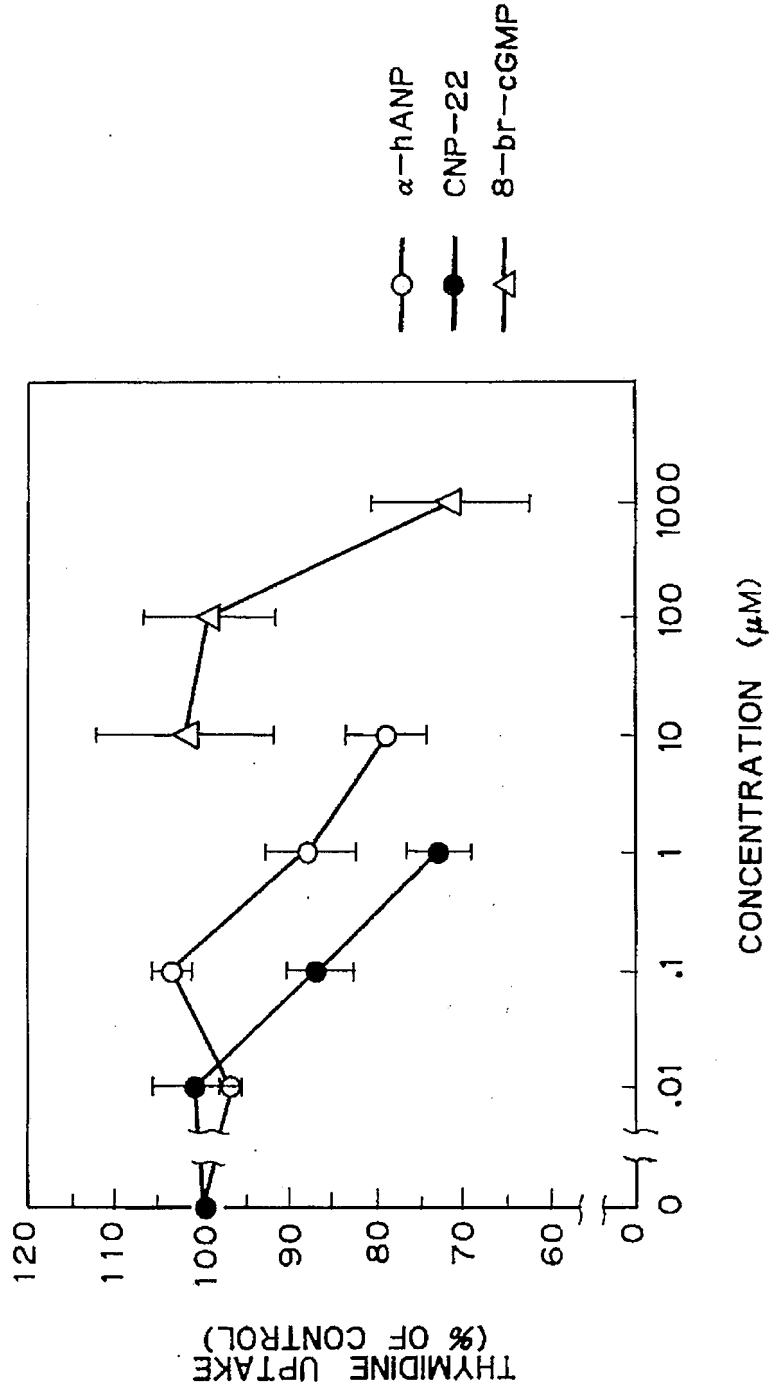


Fig. 3

INHIBITION BY α-hANP, CNP-22 AND hCNP-53 OF DNA SYNTHESIS BY CULTURED RAT VACULAR SMOOTH MUSCLE CELLS IN RESPONSE TO STIMULATION WITH PDGF (20 ng/mL)

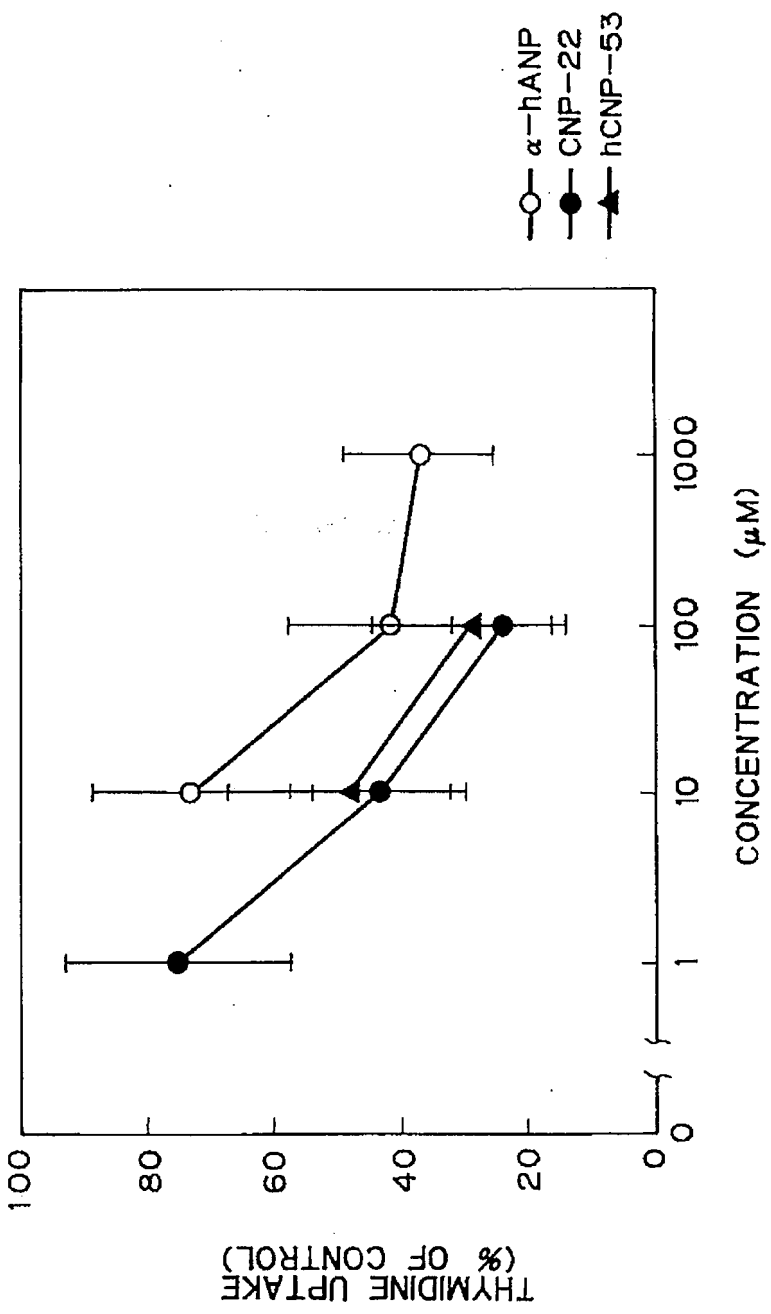
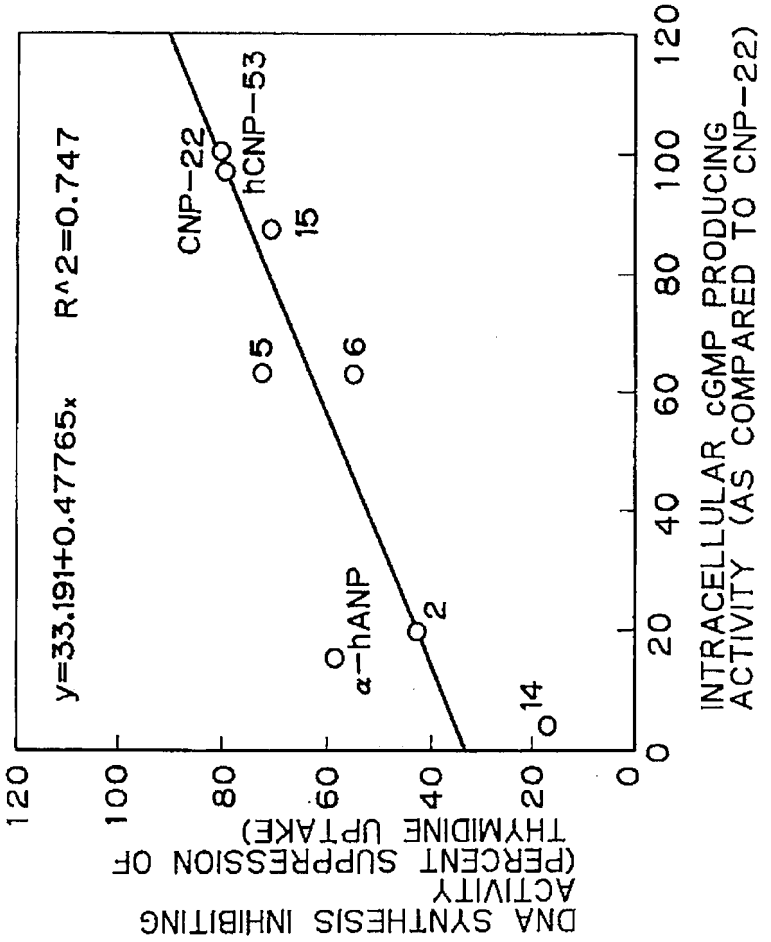


Fig. 4

CORRELATION BETWEEN THE cGMP PRODUCING ACTIVITY AND DNA SYNTHESIS INHIBITING ACTIVITY (IN THE PRESENCE OF PDGF) OF VARIOUS COMPOUNDS IN RAT VASCULAR SMOOTH MUSCLE CELLS



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US5434133 Family Legal Status Report - 11 members found

Codes shown: **All** | Positive | Negative

Jump	Publication	Title	Filed	AppINo
Status	**US5434133**	CNP analog peptides and their use	1992-01-31	US1992000828450
	JP10152445A2	PREPARATION CONTAINING CNP ANALOGUE PEPTIDE	1997-09-05	JP1997000241274
	JP06009688A2	CNP-SIMILAR PEPTIDE AND ITS USE	1991-06-28	JP1991000254066
	JP03361730B2		1997-09-05	JP1997000241274
	JP02809533B2	CNPRUIJITAIPEPUCHIDO	1991-06-28	JP1991000254066
	ES2178634T3	PEPTIDOS ANALOGOS DE CNP Y SU UTILIZACION.	1992-01-31	ES1992000101621
Status	EP0497368B1	CNP analog peptides and their use	1992-01-31	EP1992000101621
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Status	AT0219103E	CNP-ANALOGE PEPTIDE UND IHRE VERWENDUNG	1992-01-31	AT1992000101621

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2007-05-29	AS	Assignment (CHANGE OF NAME;ASSIGNOR:DAIICHI ASUBIO PHARMA CO., LTD.;REEL/FRAME:019341/0642) (CHANGE OF NAME;ASSIGNOR:DAIICHI ASUBIO PHARMA CO., LTD.;REEL/FRAME:019341/0642)(New owner: ASUBIO PHARMA CO., LTD., JAPAN 2007-04-02)
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2005-12-09	AS	Assignment (CHANGE OF NAME;ASSIGNOR:DAIICHI SUNTORY PHARMA CO., LTD.;REEL/FRAME:016871/0277) (CHANGE OF NAME;ASSIGNOR:DAIICHI SUNTORY PHARMA CO., LTD.;REEL/FRAME:016871/0277)(New owner: DAIICHI ASUBIO PHARMA CO., LTD., JAPAN 2005-10-04)
2005-12-09	AS	Assignment (CHANGE OF NAME;ASSIGNOR:DAIICHI SUNTORY PHARMA CO., LTD.;REEL/FRAME:016871/0277) (CHANGE OF NAME;ASSIGNOR:DAIICHI SUNTORY PHARMA CO., LTD.;REEL/FRAME:016871/0277)(New owner: DAIICHI ASUBIO PHARMA CO., LTD., JAPAN 2005-10-04)
2003-03-27	AS	Assignment (ASSIGNMENT OF ASSIGNORS INTEREST;ASSIGNOR:SUNTORY LIMITED;REEL/FRAME:013887/0715) (ASSIGNMENT OF ASSIGNORS INTEREST;ASSIGNOR:SUNTORY LIMITED;REEL/FRAME:013887/0715)(New owner: DAIICHI SUNTORY PHARMA CO., LTD., JAPAN 2003-03-14)
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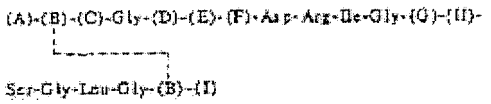
(54) CNP-SIMILAR PEPTIDE AND ITS USE

(57)Abstract:
PURPOSE: To provide a new peptide comprising a derivative of a C type sodium- urinating peptide (CNP), exhibiting an action to inhibit the multiplication of vascular smooth muscle cells, and useful as a medicine for preventing or treating arterial sclerosis, etc.
CONSTITUTION: By a solid phase peptide synthetic method, an amino acid having a protecting group is subjected to condensation and elongation reactions according to the amino acid sequence of the peptide, to the removal of all protecting groups with hydrogen fluoride, and subsequently to a disulfide-bonding reaction to provide the objective C type sodium-urinating peptide (CNP)-similar peptide represented by the formula (A is H, H-Gly, H-Lys-Gly, H-Ser-Lys-Gly, H-Leu-Ser- Lys-Gly, etc.; C is Phe, p-Cl-Phe, etc.; D is Ile, Val, etc.; E is Lys, Arg; F is Ile, Leu, etc.; G is Ser, Ala; H is Met, Gln; I is OH, Asn-OH, etc.; the dotted line is disulfide bond).



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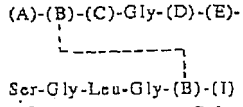
最終頁に続く

(54)【発明の名称】　ＣＮＰ類似体ペプチド及びその用途

(57)【要約】　(修正有)

【目的】　血管平滑筋細胞の増殖抑制作用を示すＣ型ナトリウム利尿ペプチド（以下ＣＮＰと略す）及びそれら＊ペプチドを有効成分とする血管平滑筋増殖抑制剤を提供する。

【構成】　一般式



〔例えば、Ｈ－Ｇｌｙ－Ｌｅｕ－Ｓｅｒ－Ｌｙｓ－Ｇｌｙ－Ｃｙｓ－Ｐｈｅ－Ｇｌｙ－Ｇｌｙ－Ａｒｇ－Ｍｅｔ－Ａｓｐ－Ａｒｇ－Ｉｌｅ－Ｇｌｙ－Ａｌａ－Ｇｌｎ－Ｓｅｒ－Ｇｌｕ－Ｌｅｕ－Ｇｌｕ－Ｃｙｓ－Ａｓｎ－Ｓｅｒ－Ｐｈｅ－Ａｒｇ－Ｔｙｒ－ＯＨ（ジスルフィド型）〕で示されるペプチド及びそれらの生理的に許容されうる酸付加物。

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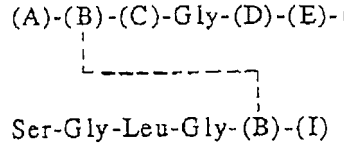
【特許請求の範囲】

【請求項1】一般式

*【化1】

*



で示される新規ペプチド及びそれらの生理的に許容されうる酸付加物。ただし　式中（A）は、H－，H－Gly，H－Lys－Gly，H－Ser－Lys－Gly，H－Leu－Ser－Lys－Gly，H－Gly－Leu－Ser－Lys－Gly，H－Ser，H－Ser－Ser，H－Arg－Ser－Ser，H－Arg－Arg－Ser－Ser，H－Leu－Arg－Arg－Ser－Ser，H－Ser－Leu－Arg－Arg－Ser－Serを表し、

（B）はH－Cys，又はPmpを、

（C）はPhe－，pCl－Phe，pF－Phe，pNO_2－Phe，またはChaを

（D）はIle，Val，Aib，tLeu，Gly，又はLeu，

（E）はLys，又はArg，

（F）はIle，Leu，又はMet，

（G）はSer，又はAla，

（H）はMet，又はGln，

（I）は－OH，－Asn－OH，－Asn－Ser－OH，－Asn－Ser－Phe－OH，－Asn－Ser－Phe－Arg－OH，または－Asn－Ser－Phe－Arg－Tyr－OHを表し、一般式中……はジスルフィド結合を示す。但し、これらの式中で1）α－hANP，2）α－hANP（7－28），3）CNP－22を除く。

【請求項2】CNP類似体ペプチドを有効成分とする平滑筋増殖抑制剤。

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明は、血管平滑筋細胞の増殖を抑制するペプチド類の合成及びその使用に関する。さらに詳しくはC型ナトリウム利尿ペプチド（以下CNPと略す）の新規誘導体の合成、CNP及びCNP誘導体の新規生理作用、さらにはこれらペプチドを有効成分とする血管平滑筋増殖抑制剤に関する。なお、本明細書において、CNP類似体ペプチドとは「請求項1」に記載した化合物、CNP－22、ヒトCNP－53、ブタCNP－53、カエルCNP、及びニワトリCNPを意味する。

【0002】

【従来の技術】近年、各種動物の心臓及び脳から、ナトリウム利尿作用及び血圧降下作用を有するペプチドが数多く発見されてきた。これらのペプチドは総称してナトリウム利尿ペプチド（natriuretic peptide；NP）と呼ばれている。従来生体から、鎖長が異なったり、アミノ酸一次配列が類似したNPが数多く単離・同定されていたが、これらは全て3つの異なるNP前駆体タンパク（prepro ANP，prepro BNP，prepro CNP）から生合成されてくることが判ってきた。

【0003】従って、現在NPはその生合成経路から次に示す3つのタイプに分類することができる。すなわち、A型NP（A type natriuretic peptide；ANP）、B型NP（B type natriuretic peptide；BNP）及びC型NP（C type natriuretic peptide；CNP）である。このうち、ANPとBNPはそれぞれ心房及び脳から単離されたため、初め、ANPは心房性ナトリウム利尿ペプチド（atrial natriuretic peptide）、BNPは脳ナトリウム利尿ペプチド（brain natriuretic peptide）とも呼ばれていた（Matsuo, H. and Nakazato, H. Endocrinol. Metab. Clin. North Amer., 16, 43, 1987；Sudoh, T. et al Nature, 332, 78, 1988）。しかし、その後の研究でANPは心房のみならず脳でも産生されていること、これと同様、BNPは脳のみならず心臓でも産生されていることが判ってきた（Ueda, S. et al., Biochem. Biophys. Res. Commun., 149, 1055, 1987；Aburaya, M. et al., Biochem. Biophys. Res. Commun., 165, 872, 1989）。また、ANPとBNPはいずれもin vivo投与で同程度の顕著なナトリウム利尿作用及び血圧降下作用を示すことが確認された。以上のことから、現在ANPとBNPはいずれも心臓から血中へ分泌されるホルモンとして働くと共に、神経伝達因子としても作動し、生体の体液量及び血圧のホメオスタシス維持に重要な役割を果たしていると考えられている。

【0004】一方、CNPはANP，BNPに続く第3のNPに分類されるペプチドで、これらのペプチドはごく最近単離され、その構造及び生合成機構が解明されてきた。最初に発見されたCNPは22アミノ酸残基より成るCNP－22とCNP－22のN－末端に31アミ

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ノ酸残基が付加されたCNP－53で、これらのペプチドはいずれもブタ脳から単離され、その構造が明らかにされた。なお、ブタ脳においてCNP－22とCNP－53はほぼ同量存在していることも明らかにされた（Sudoh, T. et al., Biochem. Biophys. Res. Commun., 168, 863, 1990；Minamino, N. etal., Biochem. Biophys. Res. Commun., 170, 973, 1990）。その後、CNP－22・CNP－53に対応するブタCNP遺伝子及びcDNAが単離され、これらの解析からCNP－22及びCNP－53はいずれも共通前駆体タンパク（prepro CNP）から生ずることが判った。また、このprepro CNPはANP及びBNP前駆体タンパク（prepro ANP, prepro BNP）とは明らかに異なっていることが判った（Tawaragi, Y. et al., Biochem. Biophys. Res. Commun., 172, 627, 1990）。

【0005】また、ブタCNP遺伝子の単離に続き、ラットCNPcDNA及びヒトCNP遺伝子が単離され、ラット及びヒトCNP前駆体タンパクの構造が明らかにされてきた。この結果、CNP－22はブタ，ヒト・ラットですべて同一アミノ酸一次配列を有すること、CNP－53はブタとラットで同一アミノ酸一次配列を有するが、ヒトとブタでは2ヶ所アミノ酸が置換していること、さらに、CNPはANP・BNPとは異なり、心臓では産生されず、脳で特異的に産生されていることが判った（Kojima, M. et al., FEBS letter, 276, 209, 1990；Tawaragi, Y. et al., Biochem. Biophys. Res. Commun., 175, 645, 1991）。さらに、現在ではカエル及びニワトリからもCNPに帰属されるペプチドが単離・同定されている（特願平02－238294：特願平02－238293）。

【0006】この様に、現在CNPは哺乳動物のみならず、鳥類及び両棲類にも存在していることが確認されてきた。しかし、CNPのNPとしての生理的役割については不明な点が多い。すなわち、CNPはそのアミノ酸一次配列がANP及びBNPと類似しており、また、in vivo投与でナトリウム利尿作用及び血圧降下作用を示すことからNPファミリーに帰属された。しかし、CNPのナトリウム利尿作用及び血圧降下作用はANP・BNPに比べ著しく弱いこと（1／50～1／100）、また、CNPの産生組織がANP及びBNPとは異なり脳に限られていることから、CNPはNPファミリーのなかでも特異的な位置を占め、その生理的役割については体液量や血圧のホメオスタシス維持以外に別な役割を果たしているのではないかと推定されていた。

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【0007】ところで、今までの研究からNPの血圧降下作用機構は、NPが血管平滑筋細胞の表面に存在するNPレセプターに結合することにより細胞内cGMP（サイクリックグアノシンモノフォスフェート）を上昇させ、このcGMPがNPの細胞内セカンドメッセンジャーとして作動し、最終的に血管弛緩を引き起こすと考えられている。実際、血管標本あるいは血管平滑筋培養細胞（VSMC）にNPを作用させると細胞内cGMPが上昇することが確かめられている。しかし、本発明者らは最近、CNPをVSMCに作用させたところ、予想に反しCNPはVSMCの細胞内cGMPをANPまたはBNPに比べ数倍強く上昇させることを見いだした（Furuya, M. et al., Biochem. Biophys. Res. Commun., 170, 201, 1990）。このことは、CNPにより誘発されたcGMPがVSMCにおいてただ単に血管弛緩作用のセカンドメッセンジャーとして作動しているばかりでなく、他の生理作用発現のメディエーターとして機能していることを示唆している。この点に関しGarg等は、ニトロプルシド、S－ニトロソN－アセチルペニシラミン等の血管弛緩剤が、ラットVSMCの系において、細胞増殖を抑制すること、さらに同じ系で8－ブロモcGMPも同様の作用を示すことを明らかにし、この増殖抑制作用が、ニトリックオキサイド（NO）ラジカルにより誘起されたcGMPによって引き起こされると結論している（Garg, U. C. et al., J. Clin. Invest., 83, 1774, 1989）。また、Kariya等は、ラビット大動脈由来の血管平滑筋培養細胞において、ANPが細胞内cGMP産生を亢進させ、この細胞の増殖を抑制させると報告している（Kariya, K. et al., Atherosclerosis, 80, 143, 1989）。これらの報告はいずれもcGMPが血管平滑筋培養細胞の増殖を抑制するメディエーターとして働いていることを強く示唆しており、また、CNPにより誘起されたcGMPも血管平滑筋培養細胞の増殖を抑制する可能性があることを示唆している。しかしながら、現在CNPが血管平滑筋培養細胞の増殖を抑制するか否かは判っていない。

【0008】一方、全てのNPは分子内S－S結合に基づき形成される17アミノ酸残基から成る環状構造を持つことが知られている。そこで、この共通環状構造を中心に、NPの構造を3つのドメイン（環外N－末端ドメイン、環内ドメイン及び環外C－末端ドメイン）に分け、CNPの構造をANP及びBNPのそれらと比較すると、CNPはANPまたはBNPと以下に述べる点が異なっていることが判る（図1参照）。すなわち、CNPのアミノ酸一次配列は、環外N－末端ドメインではANPまたはBNPと全く異なり、また、環内ドメインでは17アミノ酸残基のうちANPとは5残基、BNPとは4残基異なっていることが判る。また、CNPの環外

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C－末端ドメインの構造はANPまたはBNPと大きく異なり、CNPにはANPまたはBNPに存在するtail構造が存在しない（ANP・BNPの場合、環状構造のC－末端側にANPで5個、BNPで6個、アミノ酸残基が付加されており、この構造を便宜的にtail構造と呼ぶ）。以上述べたCNPとANPまたはBNPとの構造上の違いが、前記したCNPの特徴的薬理作用発現に関与していることは明かである。しかしながら、現在CNPのどのドメイン構造、また、どのアミノ酸一次配列がCNPの強いcGMP産生活性に直接関与しているかについては判っていない。

【0009】

【発明が解決しようとする課題】従って、本発明における課題は、まず第一に、天然から単離されたCNPに帰属されるペプチドが血管平滑筋培養細胞の増殖を抑制するか否かを明らかにすることである。第二に、CNPの構造で、CNPの強いcGMP産生活性発現に関与している最小活性構造および必須アミノ酸一次配列を明らかにすることである。また、この知見に基づき、最終的には天然由来CNPより強いcGMP産生活性を示す新規CNP誘導体を作成することである。さらに第三として、天然由来CNP及びCNP誘導体の医薬品としての利用方法を見いだすことである。

【0010】

【課題を解決するための手段】本発明者等は、まず第一の課題を明らかにする方法として、CNPが血清またはPDGF（Platelet derived growth factor）で刺激したラット血管平滑筋培養細胞のDNA合成を抑制するか否かを調べた。次に第二の課題を達成する方法として、まず、α－hANP

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（ヒトにおいて、このペプチドが主に心房から血中へ分泌されている）とCNP－22のアミノ酸配列の一部をお互いに入れ換えた誘導体及びCNP－22の環外N－末端ドメインを除去した誘導体を作成し、CNPに特徴的な強いcGMP産生活性発現にCNPのどのドメインまたはアミノ酸一次配列が関与しているかをつきとめた。また、同時にCNPの前記活性発現に必要な最小活性構造を明らかにした。次に、これらCNP誘導体の一部のアミノ酸残基を異常アミノ酸（非天然型）残基に置換することで、CNPより強いcGMP産生活性、及びDNA合成阻害活性を有するCNP誘導体を作製した。第三の課題については、本発明も含め今までに判っているCNPの生理作用及び種々の病変組織の解析から明らかにされている知見を総合的に考え、CNP及び新規CNP誘導体の医薬品としての利用方法を具体的に提示した。

【0011】具体的な説明

本発明者等は、実施例1－2に示した方法に従い、CNP－22及びヒトCNP－53（hCNP－53）がラットVSMCの細胞増殖を抑制するか否かを調べた。この結果、図2、図3及び図4に示すようにCNP－22及びhCNP－53はいずれも用量依存的に同程度の強さでラットVSMCのDNA合成を抑制することが判った。また、この作用の強さはα－hANPに比べ10倍強いことが判った。さらに表1に示すように、CNP－22は血清で刺激したVSMCの細胞数の増加をも抑制することが判った。

【0012】

【表1】

表1：CNP-22及びα-hANPの1％血清刺激下でのラット血管平滑筋細胞の増殖に対する抑制作用

化合物	用量	細胞数 (x10^3 cells/well)	[% コントロール]
生理食塩水		20.6 ± 0.2	[100]
CNP-22	5x10^{-7}M	15.0 ± 0.3 *	[72.8 ± 1.1]
α-hANP	5x10^{-7}M	17.1 ± 0.2 **	[83.0 ± 1.3]

値は平均値±標準誤差で示した（n=12）。

*；$p<0.01$, Student's t-testにて統計的に有意差あり。

**；$p<0.05$, Student's t-testにて統計的に有意差あり。

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【0013】以上のことから、CNP（CNP－22，hCNP－53）はラットVSMCに対し、細胞増殖抑制作用を示すことが初めて明らかにされた。また、この細胞増殖抑制作用の強さと細胞内cGMPの濃度との間には正の相関関係が成立していることが判った（図4）。

【0014】次に本発明者等は、以下に示す方針に従い、種々のCNP誘導体を作製し、CNPに特徴的な強いcGMP産生活性がCNPのどの構造あるいはどのアミノ酸一次配列に基づくかを調べた。前記したように、CNP－22とα－hANPの構造を環外N－末端ドメイン、環内ドメイン、及び環外C－末端ドメインに分け比較すると、CNP－22はα－hANPと以下に示す点が異なっている（図1参照）。すなわち、CNP－22のアミノ酸一次配列は環外N－末端ドメインでは全く異なり、また、環内ドメインでは17アミノ酸残基のうち5残基（CNP－22の9位ロイシン、10位リジン、11位ロイシン、16位セリン及び17位メチオニン残基。ただし、これらの残基はα－hANPにおいては、それぞれ10位グリシン、11位アルギニン、12位メチオニン、17位アラニン及び18位グルタミン残基に対応する）が異なる。また、CNP－22にはα－hANPに存在する環外C－末端ドメインが存在しない。従って、これらの構造の違いがCNPとANPとの薬理作用の違い（特にVSMCに対するcGMP産生活性の違い）に関与していることは明らかである。

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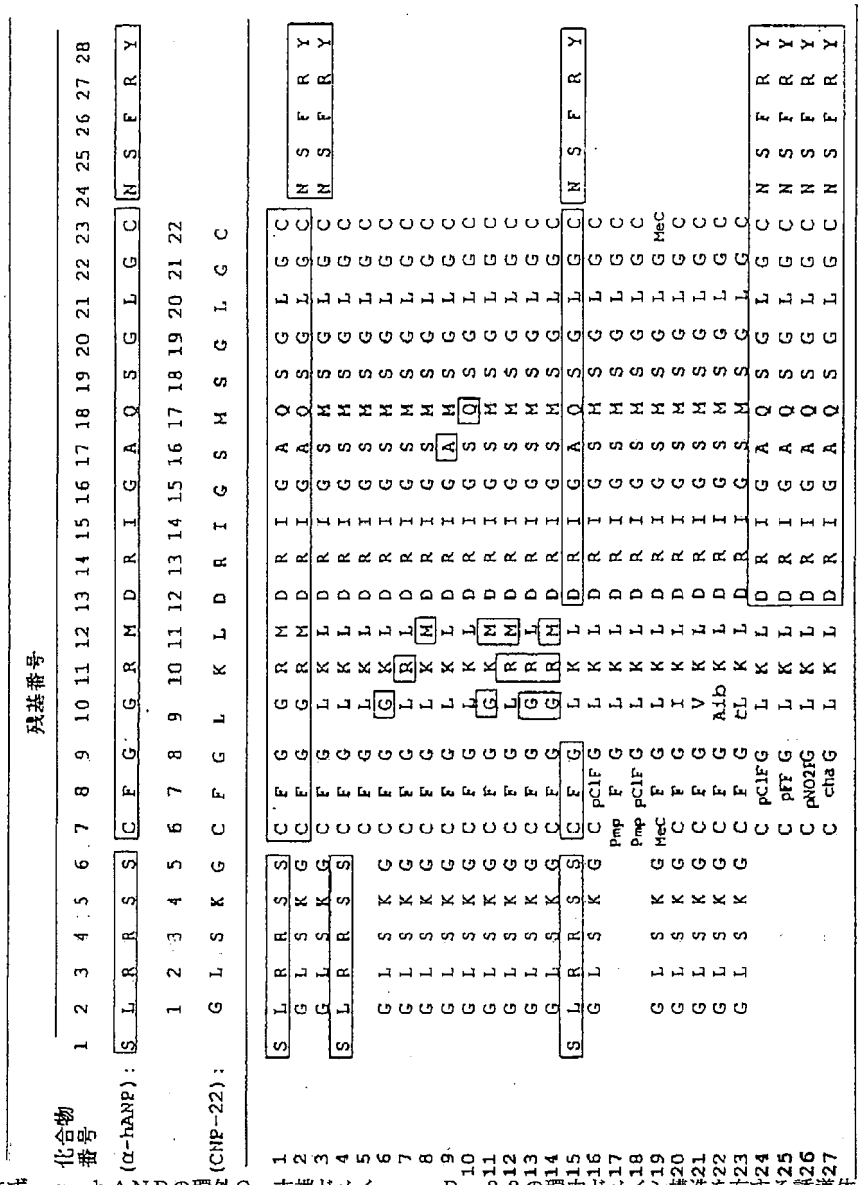
【0015】そこでまず本発明者等は、CNPに特徴的なcGMP産生活性がCNPのどのドメイン構造に基づくかを調べた。表2に本発明で合成した全ての誘導体の一次構造を示す。

【0016】

【表2】

表2：　新規CNP誘導体のアミノ酸配列



【0017】まず、α－hANPの環外C－末端ドメインを除去した誘導体、CNP－22とα－hANPの各ドメインをお互い組み換えた誘導体、及びCNP－22の環外N－末端ドメインを除去した誘導体を合成し（表2，1～5）、これら誘導体のcGMP産生活性について調べた。この結果、表3に示すように、分子内にCNP－22の環内ドメイン構造を有する誘導体3，4，及びCNP－22の環外N－末端ドメインを除去した誘導体5はいずれもCNP－22と同程度の強いcGMP産生活性を示すことが判った。

【0018】

【表3】

表3：CNP類似体の生理活性

化合物	^{3}H-チミジン取り込みの%阻害率 1)	cGMP産生活性 (1uMでの%増加率) 2)
α-hANP	58	100
CNP-22	76	621
1	NT	62
2	38	147
3	NT	667
4	NT	663
5	65	616
6	49	344
7	NT	413
8	NT	497
9	NT	707
10	NT	659
11	NT	71
12	NT	273
13	NT	58
14	17	23
15	72	559
16	NT	688
17	NT	233
18	NT	333
19	NT	38
20	NT	499
21	NT	549
22	NT	160
23	NT	NT
24	NT	719
25	NT	785
26	NT	255
27	NT	523
hCNP-53	71	458
pCNP-53	NT	524

1）PDGF（20ng/ml）で刺激したVSMCの系を用い、
各ペプチド0.1μM加えたときの阻害率を示した。

2）1μMでの各化合物のcGMP産生能を最大活性とし、α-hANPの
1μM での活性（2000fmol/400000cells）に対する比活性を求めた。

【００１９】一方、α－ｈＡＮＰの環外Ｃ－末端ドメインを除去した誘導体１及びα－ｈＡＮＰの環外Ｎ－末端ドメインをＣＮＰ－２２の環外Ｎ－末端ドメインに置き換えた誘導体２はα－ｈＡＮＰと同程度の弱いｃＧＭＰ産生活性しか示さないことが判った。

【００２０】以上のことから、ＣＮＰの強いｃＧＭＰ産生活性は、ＣＮＰ－２２の環内ドメイン構造に基づいていることが判った。また、最終的には、ＣＮＰ－２２の分子内Ｓ－Ｓ結合を開裂させた誘導体１９がほとんどｃＧＭＰ産生活性を示さないことが判り、ＣＮＰのｃＧＭＰ産生活性に関する最小活性構造は環状ＣＮＰ（６－２３）５と決定された。

【００２１】次に、ＣＮＰのｃＧＭＰ産生活性発現に、ＣＮＰの環内ドメインアミノ酸残基のうち、どのアミノ酸残基（またはアミノ酸一次配列）が重要であるかを調べた。このために、まずＣＮＰ－２２を基本骨格として、α－ｈＡＮＰとは異なるＣＮＰ－２２の環内ドメイン５アミノ酸残基（９位ロイシン、１０位リジン、１１位ロイシン、１６位セリン及び１７位メチオニン残基）を、それぞれ対応するα－ｈＡＮＰのアミノ酸残基（１０位グリシン、１１位アルギニン、１２位メチオニン、１７位アラニン及び１８位グルタミン）に置き換えた一

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残基置換体（表2，6～10）を作製し、これらのcGMP産生活性を調べた。この結果、表3に示すように、CNP－22の16位置換体9と17位置換体10は、いずれもCNP－22と同程度の強い活性を示した。これに対し、CNP－22の9，10及び11位置換体6，7，8はいずれもCNP－22に比べ活性が低下した（ただし、これらの活性はいずれもα－hANPと比べると高い）。

【0022】以上のことから、CNP－22の9位ロイシン、10位リジン及び11位ロイシン残基がCNPのcGMP産生活性発現に重要であることが判った。しかしながら、表3から明らかなように、一残基置換体（6，7，8）ではいずれも活性の低下は十分でないことから、活性に重要な残基は単一残基ではなく、CNP－22の9位ロイシン、10位リジンまたは11位ロイシン残基のうちいずれか2残基以上で構成されていることが予想された。

【0023】そこで、次にCNP－22の9位ロイシン、10位リジン、11位ロイシン残基の組み合せから得られる二残基置換体（表2，11～13）、及び三残基置換体（表2，14）を作製し、これらのcGMP産生活性を調べた。この結果、表3に示すように、三残基置換体14では予想どうり活性が激減した。また、二残基置換体はいずれも前記した一残基置換体より活性がさらに低下した。なかでも11及び13の活性低下は著しい。これらの解析から、CNP－22の環内ドメインアミノ酸一次配列で9位から11位までの配列、すなわちLeu－Lys－LeuがCNPのcGMP産生活性発現に重要であることが判った。このことは、実際α－hANPの10，11及び12位アミノ酸残基をそれぞれロイシン、リジン、ロイシン残基に置換した三残基置換体15がCNP－22とほぼ同程度の強いcGMP産生活性を示すことからも確かめられた。また、前記した実験結果からCNP－22の9位から11位までの3残基のなかで特に重要な残基を指摘することは難しいが、一残基置換体6と二残基置換体11，13はいずれもcGMP産生活性が他の一及び二残基置換体に比べ低いことから、CNPの9位ロイシン残基が特に重要と考える。言い換えれば、ANPとCNPのcGMP産生能の差は、CNP－22の9位ロイシン残基と対応するα－hANP10位グリシン残基の違いから生じると推定される。

【0024】次に、本発明者等は、本発明で明らかにされた知見及び今までにANPの構造活性相関の研究で明らかにされている知見（例えば、Minamitake, Y. et al., Biochem. Biophys. Res. Commun., 172, 971, 1990）を総合的に考え、天然由来NP（ANPまたはCNP）より強いcGMP産生活性及びDNA合成阻害活性を有するCNP誘導体の作製を試みた。

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【0025】本発明者等は、まず、CNP－22の9位ロイシン残基に着目し、CNP－22を基本骨格として9位ロイシン残基をイソロイシン、バリン、α－アミノイソ酪酸、あるいはt－ロイシン残基に置換した誘導体（表2，20～23）を作製した。次に、CNP（6－22）5を基本骨格として、6位システイン残基をペンタシクロメルカプトプロピオニル基に置換した誘導体、7位フェニルアラニン残基をp－クロローフェニルアラニン残基に置換した誘導体、及び、6位及び7位をそれぞれペンタシクロメルカプトプロピオニル及びp－クローフェニルアラニン残基で同時に置換した誘導体を合成した（表2，16，17，18）。さらに、［Leu10，Lys11，Leu12］α－hANP（7－28）を基本骨格として、8位フェニルアラニン残基をp－クロローフェニルアラニン、p－フルオローフェニルアラニン、p－ニトローフェニルアラニン、あるいはシクロヘキシルアラニン残基で置換した誘導体を作製した（表2，24～27）。また、これら誘導体のcGMP産生を調べた。

【0026】この結果、第3表に示すように、まず、CNP－22を基本骨格とする誘導体では、20及び21がCNP－22とほぼ同程度の活性を示すことが判った。次に、CNP（6－22）を基本骨格とする誘導体では、16がCNP－22より強い活性を示すことが判った。さらに、［Leu10，Lys11，Leu12］α－hANP（7－28）を基本骨格とする誘導体では、15，24，25及び27がいずれもα－hANPに比べ4～6倍強い活性を示した。なかでも、誘導体24及び25の活性はCNP－22より強いことが判った。

【0027】これらの結果から、CNP－22、CNP（6－22）及び［Leu10，Lys11，Leu12］α－hANP（7－28）の一部のアミノ酸残基を異常アミノ酸（非天然型）残基に置換することで、CNP－22あるいはα－hANPより強いcGMP産生活性を示す誘導体を作製できることが判った。

【0028】次に、CNP及びCNP誘導体の医薬品への利用方法について具体的に述べる。現在までに、血管平滑筋細胞の異常増殖に起因する疾患が種々報告されている。例えば、冠動脈の再狭窄は経皮経管腔冠動脈形成術（PTCA）に成功した患者の約30％に発生し、この原因はほとんどの場合血栓形成ではなく、動脈平滑筋細胞の異常増殖であることが知られている。また、これと同様な再狭窄は動脈バイパスなどを含め移植された組織の血管でも生ずることが知られている。さらに、血管平滑筋の増殖は動脈硬化症の患者の血管でもしばしば見いだされている。しかしながら、今までにこれら血管平滑筋の増殖に起因する疾患に対し有効な治療薬は見いだされておらず、現在その開発が望まれている。

【0029】一方本発明者等は、本発明において、CN

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Pが血管平滑筋細胞の増殖を強く抑制することを初めて明らかにした。また、この作用の強さと、cGMP産生活性の強さとの間には正の相関関係が成立することを明らかにした。さらに、天然由来ANPまたはCNPより強いcGMP産生活性を示す誘導体を作製することに成功した。

【0030】以上を総合的に考えると、血管平滑筋細胞に対し、強いcGMP産生活性を示すCNP及びCNP誘導体は、再狭窄及び動脈硬化など血管平滑筋細胞の異常増殖が原因となる疾患に対し、有効な治療薬または予防薬となりうる。以上、本発明において、まずCNPが血管平滑筋細胞の異常増殖を強く抑制することを明らかにし、この作用の強さとcGMP産生活性の強さとの間には正の相関関係が成立することを見いだした。

【0031】次に、CNPの新規誘導体を種々合成することにより、CNPのVSMCに対するcGMP産生活性に関する最小活性構造はCNP（6－22）であることを見いだした。また、天然由来ANPまたはCNPより強いcGMP産生活性を示す新規CNP誘導体の合成に成功した。さらに、CNP及びCNP誘導体が、再狭窄及び動脈硬化など血管平滑筋細胞の異常増殖が原因となる疾患に対し、有効な治療薬または予防薬となりうることを見いだし、本発明を完成させた。なお、本発明においては、具体的実施例として第2表に示したCNP誘導体について述べるが、本発明の知見からすれば、VSMCに対し強いcGMP産生活性を示す誘導体の作製は、すでに構造が明らかにされている他のNPに適応できる。

【0032】本発明に関するペプチドは、無機酸、例えば塩酸、硫酸、リン酸、あるいは有機酸、例えばギ酸、酢酸、酪酸、コハク酸、クエン酸等の酸付加塩に転換できる。本発明に関するペプチドの製造は、標準的な化学合成法、あるいは組み換えDNA法（ただし非天然形アミノ酸残基を含む誘導体は除く）を用いて行うことができる。化学合成法の一般的総書としては、例えば「生化学実験講座Ⅰ　タンパク質の化学ⅣV　第ⅡⅠ部207～495頁」（東京化学同人）、「ペプチド合成の基礎と実験・泉屋信夫他共著」（丸善）、また「ペプチドケミストリー1984，229～234頁，235～240頁，及び241～246頁・泉屋編集」（蛋白研発行）などがあり、これらに合成法が詳細に記載されている。また、組み換えDNA法による製造法としては、例えば「遺伝子操作1990、蛋白質核酸酵素臨時増刊2613～2619頁、高浪満・木村光編集」（共立出版）があり、これに基本的製造法が記載されている。

【0033】本発明に関するペプチドは、上記文献に記載されている化学合成法に準じて合成した。すなわち、保護基のついたアミノ酸を固相法と称せられる方法で縮合・延長させ、弗化水素で全保護基を除去した後、ジスルフィド結合反応を経て製造した。前記の方法等で得られた粗ペプチドは、イオン交換カラムクロマト、逆相カラムクロマト等、常用される精製法の組み合せにより純品として得られる。本発明の医薬組成物は、本発明に関するペプチドの遊離型としても、あるいはその薬理学的に許容される酸付加塩としても投与することができる。

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【0034】本発明に関するペプチドもしくはその薬理学的に許容される酸付加塩は、公知の薬理学的に許容される担体、賦型剤、希釈剤などと混合して、ペプチド性医薬に一般に使用されている投与方法、すなわち非経口投与法（静脈内投与、筋肉内投与、皮下投与など）によって投与するのが望ましいが、有効成分である本ペプチドをリポソームあるいはポリアミドなどに包容することで、消化管内で分解されにくいマイクロカプセル製剤として経口投与することも可能である。また、坐剤、点鼻スプレー、点眼剤、舌下錠剤等の形態でそれぞれ直腸、鼻腔内、眼、舌下などの粘膜から吸収される投与方法も可能である。

【0035】本発明の医薬組成物の投与量は、疾患の種類、患者の年齢、体重、症状の程度及び投与経路などによっても異なるが、一般的に1日当り0．01mg／body～10mg／bodyの範囲で投与可能であり、0．05mg／body～1mg／bodyの範囲で投与するのが好ましい。

【0036】本明細書において特に標記のないアミノ酸はL－体であり、試薬類を含め下に示される略号を用いた。

【0037】Asp：L－アスパラギン
Asp（OcHex）：β－シクロヘキシルアスパラギン酸
Ser：L－セリン
Ser（Bzl）：O－ベンジル－L－セリン
Gln：L－グルタミン
Gly：グリシン
Ala：L－アラニン
Cys：L－システイン
Cys（4MeBzl）：4－メチルベンジル－L－システイン
Met：L－メチオニン
Ile：L－イソロイシン
Leu：L－ロイシン
t－Leu：L－ターシャルロイシン
Tyr：L－チロシン
Tyr（BrZ）：0－2－ブロモベンジルオキシカルボニル－L－チロシン
Phe：L－フェニルアラニン
Arg：L－アルギニン
Arg（Tos）：G－トシル－L－アルギニン
pClPhe：パラクロロ－L－フェニルアラニン
Pmp：ペンタシクロメルカプトプロピオン酸
Aib：アミノイソ酪酸

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Ｌｙｓ：Ｌ－リジン
Ｂｏｃ－：ｔ－ブチルオキシカルボニル
ＴＦＡ：トリフルオロ酢酸
ＮＭＰ：Ｎ－メチルピロリドン
ＤＭＳＯ：ジメチルスルホキシド
ＨＯＢｔ：Ｎ－ヒドロキシベンゾトリアゾール
ＤＩＥＡ：ジイソプロピルエチルアミン
ＤＣＣ：ジシクロヘキシルカルボジイミド
最終物の純度検定を、下に示す薄層クロマトグラフィー、分析用高速液体クロマトグラフィー、及びアミノ酸分析にて実施した。

【００３８】薄層クロマトグラフィー
担体：シリカゲル６０　Ｆ－２５４（メルク）
展開溶媒：
Ｒｆ１　ｎ－ブタノール：酢酸：ピリジン：水＝４：１：１：２
Ｒｆ２　ｎ－ブタノール：酢酸：ピリジン：水＝３０：２０：６：２４
分析用高速液体クロマトグラフィー
機器：島津ＬＣ－６Ａシステム
カラム：ＹＭＣ－Ｐａｃｋ　Ａ－３０２　ＯＤ５　４．６φｘ１５０ｍｍ
展開溶媒：１８％　CH_3CN／０．１％　ＴＦＡから　６０％　CH_3CN／０．１％ＴＦＡまでの３０分リニアグラジエント
アミノ酸分析
機器：日立アミノ酸分析機８３５型
【００３９】
【実施例】
実施例１．生物活性の測定
１－１：ｃＧＭＰ産生活性の測定
本発明で合成した化合物の上記活性をＨｉｒａｔａら（Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ｒｅｓ．Ｃｏｍｍｕｎ．，１２８　５３８，１９８５）、及びＳｃａｒｂｏｒｏｕｇｈら（Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．，２６１　１２９６０，１９８６）の方法に従って測定した。用いた細胞は、ラット大動脈由来の血管平滑筋培養細胞（以下ＶＳＭＣと略す）である。１０－９～１０－６Ｍのα－ｈＡＮＰ、及びペプチドをＶＳＭＣと共にインキュベートし、産出したｃＧＭＰ量を、ｃＧＭＰラジオイムノアッセイにて測定した。α－ｈＡＮＰによる最大反応値を１００％とし、各ペプチドの最大反応率を求め活性の指標とした。

【００４０】１－２：細胞増殖抑制活性の測定
細胞増殖抑制活性は、前記ＶＳＭＣを用いてＫａｒｉｙａ等の方法に従い（Ａｔｈｅｒｏｓｃｌｅｒｏｓｉｓ，８０，１４３－１４７，１９９０）、［^{3}Ｈ］チミジンの細胞内への取り込みを指標にＤＮＡ合成阻害率で測定した。すなわち、静止期に同調させた細胞を１％血清あるいは２０ｎｇ／ｍｌのＰＤＧＦ（血小板由来成長因子）存在下、各検体を加え３７℃にて１４時間インキュベートした。次に、この細胞に３７ｋＢｑ／ｍｌの［^{3}Ｈ］チミジンを加えてさらに４時間インキュベートし、この間細胞内に取り込まれた［^{3}Ｈ］チミジン量を測定した。各検体のＤＮＡ合成阻害活性はペプチド非存在下で１％血清あるいはＰＤＧＦのみを加えたときの［^{3}Ｈ］チミジンの取り込み量を１００％とし、これに対する抑制率で示した。測定結果を図２、図３及び表３に示す。また、ＣＮＰ－２２に関しては、ＶＳＭＣを１％血清存在下、ＣＮＰ－２２あるいはα－ｈＡＮＰを加え４日間培養し、血球計算盤を用い細胞数を数えた。この結果を表１に示す。

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【００４１】実施例２．ＣＮＰ誘導体の合成
本発明ペプチドは、すべてアプライドバイオシステム社製ペプチド合成機４３１型を用い固相法にて作成した。代表例として表２に示す化合物２と１９の合成を示す。
２－１：化合物番号２；
Ｈ－Ｇｌｙ－Ｌｅｕ－Ｓｅｒ－Ｌｙｓ－Ｇｌｙ－Ｃｙｓ－Ｐｈｅ－Ｇｌｙ－Ｇｌｙ－Ａｒｇ－Ｍｅｔ－Ａｓｐ－Ａｒｇ－Ｉｌｅ－Ｇｌｙ－Ａｌａ－Ｇｌｎ－Ｓｅｒ－Ｇｌｕ－Ｌｅｕ－Ｇｌｕ－Ｃｙｓ－Ａｓｎ－Ｓｅｒ－Ｐｈｅ－Ａｒｇ－Ｔｙｒ－ＯＨ（ジスルフィド型）の合成
０．７ｇ（０．５ｍｍｏｌ）のＢｏｃ－Ｔｙｒ（Ｂｒ－Ｚ）－Ｏ－ＣＨ２－ＰＡＭ樹脂より出発し、６０％ＴＦＡによる脱Ｂｏｃ、ＤＩＥＡによる中和、及びＤＣＣ／ＨＯＢｔによる保護アミノ酸縮合を順次繰り返し、約２．１ｇの保護ペプチド樹脂を得た。このものをパラ・クレゾール３ｍｌ）存在下、－２℃で６０分間、ＨＦ（１７ｍｌ）処理した。遊離ペプチドを５０ｍｌのＴＦＡで抽出後濃縮し、エーテルを加え、８００ｍｇの粗ペプチドを得た。このものを３２ｇの飽和ウレア水に溶かし、フェリシアン化カリウム（１４７ｍｇ，４４．８μｍｏｌ）を含む飽和ウレア水（２８８ｍｌ，ｐＨ７．４）中に撹拌下、滴下した。滴下終了後、反応液を酢酸でｐＨ５とした後、１Ｎ　ＡｃＯＨで平衡化したＡＧ３－Ｘ４Ａ（１０ｍｌ，Ｃｌ－型）と、ＨＰ－２０（１５０ｍｌ）の連結カラムに添加した。１Ｎ　ＡｃＯＨ（５００ｍｌ）で洗浄後、ＨＰ－２０に吸着したペプチドを８０％CH_3CN／１Ｎ　ＡｃＯＨで溶出した。ペプチドを含む画分を濃縮し、凍結乾燥して粗環状ペプチド（７５０ｍｇ）を得た。

【００４２】次に、水で平衡化したイオン交換カラム（ＣＭ－２ＳＷ，２φｘ１５ｃｍ）に添加し、水から０．５Ｍ　ＮＨ４ＯＡｃ（ｐＨ７．２）への６０分間のリニヤグラジエントでペプチドを溶出させた。主画分を集め０．１％ＴＦＡで初期化した逆相Ｃ１８カラム（ＹＭＣ－Ｐａｃｋ　Ｄ－ＯＤＳ　２φｘ２５ｃｍ）に添加し、３０％CH_3CN／０．１％ＴＦＡから６０％CH_3CN／０．１％ＴＦＡへの６０分間のリニヤグラジエントをかけ、１０ｍｌ／ｍｉｎで溶出させた。９７％以

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上の純度をもつ画分を集め、凍結乾燥し１５０ｍｇの目的物（２）を得た。本実施例に基づき、１９を除く他の誘導体を作成した。

【００４３】２－２：化合物番号１９；

Ｈ－Ｇｌｙ－Ｌｅｕ－Ｓｅｒ－Ｌｙｓ－Ｇｌｙ－Ｃｙｓ（Ｍｅ）－Ｐｈｅ－Ｇｌｙ－Ｌｅｕ－Ｌｙｓ－Ｌｅｕ－Ａｓｐ－Ａｒｇ－Ｉｌｅ－Ｇｌｙ－Ｓｅｒ－Ｍｅｔ－Ｓｅｒ－Ｇｌｙ－Ｌｅｕ－Ｇｌｙ－Ｃｙｓ（Ｍｅ）－ＯＨ

の合成

ＣＮＰ（６．０ｍｇ）の水溶液（５ｍｌ）にジチオスレイトール（１０ｍｇ）を加え、１０％アンモニア水でｐＨ８．５とした後、室温で３０分間放置した。逆相Ｃ１８カラム（ＹＭＣ－Ｐａｃｋ　Ｄ－ＯＤＳ　２φｘ２５ｃｍ）に反応液を添加後、実施例１－１に準じ、CH_3CNのグラジエント溶出によりペプチドを単離、凍結乾燥して５．７ｍｇ（２．６μｍｏｌ）の還元型ＣＮＰを得た。このものを水（３ｍｌ）に溶かし、１．６ｍｇ（７．３μｍｏｌ）の４－ニトロベンゼンスルホン酸メチルを含むアセトニトリル溶液（０．５ｍｌ）を、上記水溶液に加え、２時間室温に放置した。原料の消失をＨＰＬＣで確認後、逆相Ｃ１８カラムでペプチドを分離し、４．５ｍｇの目的物（１９）を得た。

【００４４】

【発明の効果】本発明では、まずＣＮＰの生理活性作用に関して、ＣＮＰは血管平滑筋細胞に対し、強い細胞増殖抑制活性を有することが初めて明らかにされた。また、この作用の強さはα－ｈＡＮＰに比べ１０倍強いことがわかった。さらに、この作用の強さと細胞内ｃＧＭｐの濃度との間には正の相関関係が成立することが見いだされた。次に、ＣＮＰの構造活性相関に関して、ＣＮＰのｃＧＭＰ産生活性に関する最小活性構造は環状ＣＮＰ（６－２３）５であることがわかった。また、ＣＮＰの環内ドメインアミノ酸一次配列でＣＮＰ－２２の９位

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から１１位までの配列（Ｌｅｕ－Ｌｙｓ－Ｌｅｕ）がＣＮＰのｃＧＭＰ産生活性発現に重要であることがわかった。さらに、新規ＣＮＰ誘導体の合成に関して、ＣＮＰ－２２、ＣＮＰ（６－２２）及び［Ｌｅｕ１０、Ｌｙｓ１１，Ｌｅｕ１２］α－ｈＡＮＰ（７－２８）の一部のアミノ酸残基を異常アミノ酸（非天然型）残基に置換することでＣＮＰ－２２あるいはα－ｈＡＮＰより強いｃＧＮＰ産生活性を示す誘導体を作製できることがわかった。以上のことから、血管平滑筋細胞に対し、強いｃＧＭＰ産生活性ならびに細胞増殖抑制活性を示すＣＮＰ及びＣＮＰ誘導体は、再狭窄及び動脈硬化など血管平滑筋細胞の異常増殖が原因となる疾患に対し、極めて有効な治療薬または予防薬としてその有用性が期待できる。なお、本発明で合成した誘導体の中で、異常アミノ酸（非天然型）残基を含んでいる誘導体は、ｉｎ　ｖｉｖｏに投与した場合、生体内（血中及び細胞表面）に存在するプロテアーゼに対し抵抗性を示すことが考えられる。従って、これらの誘導は、たとえＣＮＰ－２２またはα－ｈＡＮＰに比べそのｃＧＭＰ産生活性が低下していても、血中半減期は異常アミノ酸を含まないＣＮＰ類似体に比べ長くなることが考えられ、この観点から有用性が期待できる。

【図面の簡単な説明】

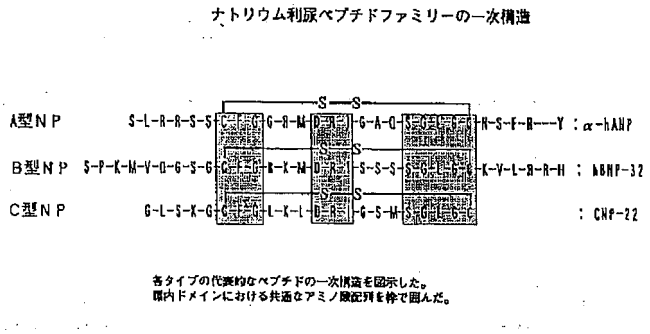
【図１】Ａ型ＮＰ、Ｂ型ＮＰ、及びＣ型ＮＰそれぞれに属する代表的なナトリウム利尿ペプチドの一次構造を示す図である。

【図２】α－ｈＡＮＰ、ＣＮＰ－２２のＤＮＡ合成阻害作用を示すグラフである。

【図３】α－ｈＡＮＰ、ＣＮＰ－２２、ｈＣＮＰ－５３のＤＮＡ合成阻害作用を示すグラフである。

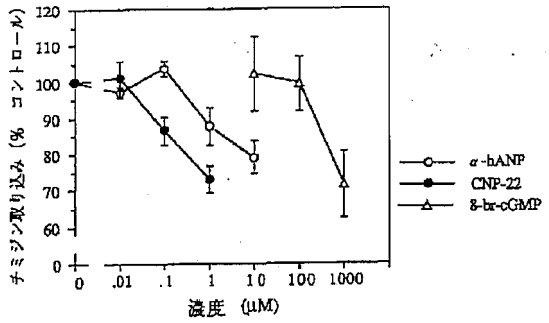
【図４】ＣＮＰ類似体ペプチドのｃＧＭＰ産生活性とＤＮＡ合成阻害活性の相関関係を示すグラフである。

【図１】



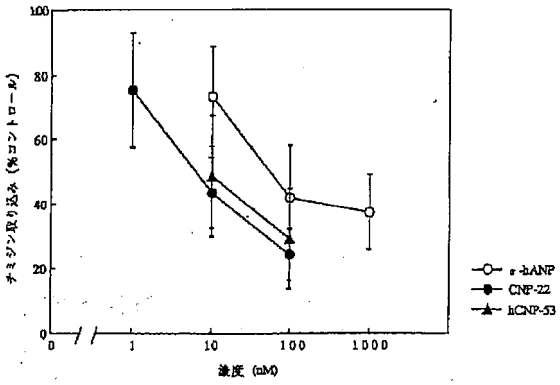
【図２】

α-hANP, CNP-22及び8-br-cGMPの１％血清刺激による
ラット血管平滑筋培養細胞DNA合成に対する阻害作用



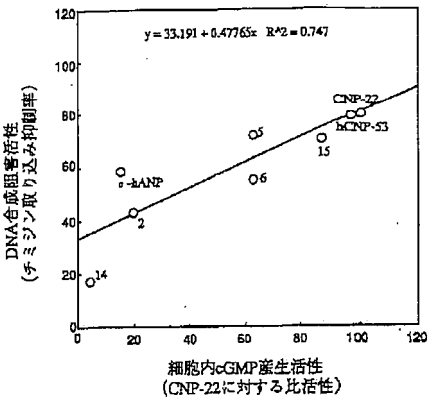
【図３】

α-hANP, CNP-22及びhCNP-53のPDGF(20ng/ml)刺激による
ラット血管平滑筋細胞DNA合成に対する阻害作用



【図４】

各化合物のラット血管平滑筋細胞におけるcGMP産生活性と
DNA合成阻害活性(PDGF存在下)の相関関係



フロントページの続き

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文献 1

Dwarfism and early death in mice lacking C-type natriuretic peptide

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Longitudinal bone growth is determined by endochondral ossification that occurs as chondrocytes in the cartilaginous growth plate undergo proliferation, hypertrophy, cell death, and osteoblastic replacement. The natriuretic peptide family consists of three structurally related endogenous ligands, atrial, brain, and C-type natriuretic peptides (ANP, BNP, and CNP), and is thought to be involved in a variety of homeostatic processes. To investigate the physiological significance of CNP *in vivo*, we generated mice with targeted disruption of CNP (*Nppc*$^{-/-}$ mice). The *Nppc*$^{-/-}$ mice show severe dwarfism as a result of impaired endochondral ossification. They are all viable perinatally, but less than half can survive during postnatal development. The skeletal phenotypes are histologically similar to those seen in patients with achondroplasia, the most common genetic form of human dwarfism. Targeted expression of CNP in the growth plate chondrocytes can rescue the skeletal defect of *Nppc*$^{-/-}$ mice and allow their prolonged survival. This study demonstrates that CNP acts locally as a positive regulator of endochondral ossification *in vivo* and suggests its pathophysiological and therapeutic implication in some forms of skeletal dysplasia.

There are two major mechanisms of bone formations, membranous and endochondral ossifications. The former occurs when mesenchymal precursor cells directly differentiate into bone-forming osteoblasts, a process by which all flat bones are formed. The latter involves the conversion of an initial cartilage template into bone and is responsible for the formation of long bones and vertebrae.

The natriuretic peptide system consists of a family of three structurally related endogenous ligands, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (1), and three membrane-bound receptors, two of which are guanylyl cyclase (GC)-coupled receptors (GC-A and GC-B) that mediate the biological actions of the ligands, and one of which is a biologically silent receptor (C-receptor) implicated in the metabolic clearance of the ligands (2, 3). ANP and BNP are cardiac hormones that are produced predominantly by the atrium and ventricle, respectively (4–6), and are thought to play important roles in the regulation of cardiovascular homeostasis, primarily through GC-A (7, 8). On the other hand, CNP occurs in a wide variety of central and peripheral tissues (9–12) and may act locally as an autocrine/paracrine regulator through GC-B (7, 8).

We have created transgenic mice overexpressing BNP under the control of the liver-specific serum amyloid P component promoter and demonstrated that they exhibit blood pressure reduction (13). They also show marked skeletal overgrowth accompanied by the activation of endochondral ossification (14). It has been also reported that C-receptor-deficient mice (*Npr3*$^{-/-}$ mice) show skeletal abnormalities similar to those seen in transgenic mice overexpressing BNP (15, 16). These observations suggest that natriuretic peptides can affect endochondral ossification. In this context, previous studies have revealed no such skeletal abnormalities in ANP (*Nppa*)-deficient mice (17) or BNP (*Nppb*)-deficient mice (18), indicating that neither ANP nor BNP is involved in endochondral ossification under physiological conditions. In this study, we generated mice with targeted disruption of CNP (*Nppc*$^{-/-}$ mice) and demonstrated that they show severe dwarfism and early death as a result of impaired endochondral ossification. Targeted expression of CNP in the growth plate chondrocytes can rescue the skeletal defect of *Nppc*$^{-/-}$ mice and allow their prolonged survival. This study demonstrates that CNP is a bona fide endogenous natriuretic peptide in the bone, where it activates endochondral ossification.

Materials and Methods

Gene Targeting and Generation of Transgenic Mice. The 129/Sv mouse *Nppc* was isolated from a 129/Sv mouse genomic library in λFixII (Stratagene, La Jolla, CA). A targeting vector was constructed, in which exons 1 and 2 of the 129/Sv mouse *Nppc* that encode the entire coding sequences of mouse preproCNP were replaced by the neomycin resistance gene (Fig. 1*a*). The targeting vector was introduced into embryonic stem cells by electroporation (18). Double selection in G418 and ganciclovir produced seven homologously recombinant embryonic stem cell clones that were analyzed by Southern blot analysis with the 5′ and 3′ external probes indicated (Fig. 1 *a* and *b*). Male chimeras derived from two independent clones with germ-line transmission of the disrupted allele were bred to C57BL/6J or 129/SvJ females.

Generation of transgenic mice (Tg mice) with targeted expression of CNP in the growth plate chondrocytes under the control of the mouse pro-α1(II) collagen (*Col2a1*) promoter [provided by R. de Crombrugghe at the M. D. Anderson Cancer Center, Houston, TX (19)] will be reported elsewhere (A.Y., Y.K., H.C., T.M., Y.O., and Kazuwa Nakao, unpublished observations). The transgene expression was detected only in the chondrocytes (19). To perform genetic rescue of *Nppc*$^{-/-}$ mice, Tg mice were mated with *Nppc*$^{+/-}$ mice, and F1 offspring heterozygous for the transgene and for the *Nppc* allele ablation were bred to generate *Nppc*$^{-/-}$ mice with the transgene expression (Tg/*Nppc*$^{-/-}$ mice). The care of the animals and all experiments were conducted in accordance with the institutional guidelines of Kyoto University Graduate School of Medicine.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; GC-B, guanylyl cyclase-B; Tg, transgenic; *Nppc*, *Npr2*, *Col2a1*, *Gapd*, mouse genes for CNP, GC-B, pro-α1(II) collagen, and glyceraldehyde-3-phosphate dehydrogenase, respectively.

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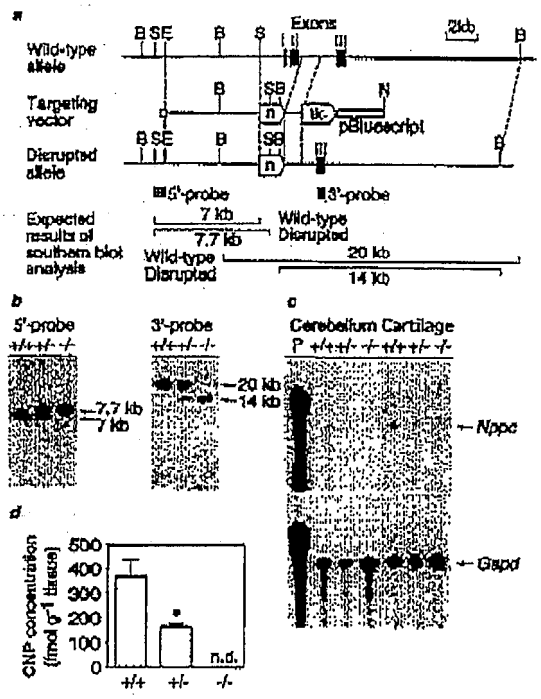

Fig. 1. Targeted disruption of the mouse *Nppc*. (*a*) Restriction maps of the wild-type 129/Sv mouse *Nppc* allele, targeting vector, and the predicted disrupted allele. Closed boxes indicate exons (I–III). Locations of 5′ and 3′ external probes are shown as hatched bars. B, *Bam*HI; S, *Sph*I; E, *Eco*RI; N, *Not*I; tk, Herpes simplex virus thymidine kinase gene; n, neomycin resistance gene. (*b*) Southern blot analysis of genomic DNAs from F2 offspring with 5′ and 3′ probes upon digestion with *Sph*I and *Bam*HI, respectively. (*c*) RNase protection analysis of *Nppc* and *Gapd* transcript in the cerebellum and tibial epiphyseal cartilage. The cerebellum and tibial cartilage are obtained from mice at 20 weeks and 7 days of age, respectively. Ten micrograms and 1 μg of total RNA were used to analyze *Nppc* and *Gapd* transcripts, respectively. (*d*) Cerebellar CNP concentrations at 20 weeks of age ($n = 4$). n.d., not detectable. *, $P < 0.05$ vs. $Nppc^{+/+}$ mice.

RNA and Peptide Analysis. Total RNA was extracted from various tissues from $Nppc^{+/+}$, $Nppc^{+/-}$, and $Nppc^{-/-}$ mice. In the cerebellum and epiphyseal cartilage, *Nppc* mRNA expression was assessed by RNase protection assay. Ten micrograms and 1 μg of total RNA were used to analyze *Nppc* and glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) transcripts, respectively. ^{32}P-labeled antisense *Nppc* and *Gapd* riboprobes were generated from a 5′-rapid amplification of cDNA ends-based 129/Sv mouse *Nppc* cDNA fragment and mouse *Gapd* cDNA fragment (a gift from M. B. Prystowsky at the Albert Einstein College of Medicine), respectively. In other tissues, *Nppc* and *Gapd* mRNA expressions were assessed by reverse transcription (RT)-PCR and Southern blot analysis (*Nppc*: sense primer, 5′-AAAAAGGGTGACAAGACTCCAGGCAG-3′; antisense primer, 5′-GGTGTTGTGTATTGCCAGTA-3′; antisense probe, 5′-CCTTC-TTGTTGCCGCCTTTGAT-3′; *Gapd*: sense and antisense primers, a mouse G3PDH control amplimer set, CLONTECH; antisense probe, 5′-GCCTTGACTGTGCCGT-TGAATTTGCCGTGA-3′). Cerebellar CNP concentrations were determined by a RIA for CNP (9).

Skeletal Preparation and Histology. Skeletal preparation and histological analysis were performed as described (14). Briefly, mice were killed, skinned, eviscerated, and subjected to soft x-ray analysis (23 kVp, 5 mA for 1 min; Softron Type SRO-M5, Softron, Tokyo). Bones from 7-day-old mice were fixed in 4% paraformaldehyde in 0.01 M PBS (pH 7.4), decalcified in 10% EDTA, and embedded in paraffin. Five-micrometer-thick sections were sliced and stained with Alcian blue (pH 2.5) and hematoxylin/eosin. The slices were also analyzed for apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling assay according to the manufacturer's protocol (Takara Shuzo, Shiga, Japan).

Detection of BrdUrd-Labeled Cells. Mice at 1 week of age were injected intraperitoneally with BrdUrd (100 μg/g body weight) and were killed 6 h later. Tibiae were fixed in 4% paraformaldehyde in 0.01 M PBS (pH 7.4), decalcified in 10% EDTA, and embedded in paraffin. Detection of BrdUrd-positive cells in the growth plate was performed by BrdUrd labeling and with a detection kit II (Roche Diagnostics).

***In Situ* Hybridization.** Digoxigenin-labeled antisense and sense riboprobes were obtained from reverse transcription–PCR products for *Nppc*, rat GC-B (nucleotides 762-1394 of rat GC-B cDNA; GenBank M26896), mouse pro-α_1(I) collagen (*Col1a1*) (nucleotide 693-1139 of *Col1a1* cDNA; GenBank M14423) and rat pro-α_1(X) collagen (a gift from B. R. Olsen at Harvard Medical School, Boston, MA), mouse pro-α_1(II) collagen (*Col2a1*) (a gift from Y. Yamada at the National Institutes of Health, Bethesda, MD), and mouse Indian hedgehog (*Ihh*) (a gift from K. Lee at Massachusetts General Hospital, Boston, MA) cDNA fragments with the use of a digoxigenin RNA labeling kit (Roche Diagnostics). Tibiae from 7-day-old mice were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde, 1 mM $CaCl_2$ in 0.1 M phosphate buffer (pH 7.2) for 24 h, embedded in paraffin, and sliced into 5-μm-thick sections. *In situ* hybridization analysis was performed as described (20).

cGMP Assay. The tail bone cGMP concentrations in 7-day-old mice were determined by a RIA for cGMP as described (21).

Statistical Analysis. Data are expressed as means ± SE. The statistical significance of differences in mean values was assessed by Student's *t* test. The difference in survival rates among genotypes was assessed by Kaplan–Meier analysis.

Results and Discussion

Generation of $Nppc^{-/-}$ Mice. To investigate the physiological significance of CNP *in vivo*, we generated mice with a disrupted *Nppc* allele by gene targeting in 129/Sv mouse-derived embryonic stem cells (Fig. 1*a*). Male chimeras with germ-line transmission of the disrupted allele were bred to C57BL/6J and 129/SvJ females, and $Nppc^{+/+}$ mice (wild type), $Nppc^{+/-}$ mice (heterozygous for the disrupted allele), and $Nppc^{-/-}$ mice (homozygous for the disrupted allele) were obtained (Fig. 1*b*). The *Nppc* mRNA levels were decreased by ≈50% in the cerebellum and tibial epiphyseal cartilage from $Nppc^{-/-}$ mice relative to those of $Nppc^{+/+}$ mice (Fig. 1*c*). Cerebellar CNP concentrations were also decreased in $Nppc^{+/-}$ mice relative to $Nppc^{+/+}$ mice ($P < 0.05$, $n = 4$) (Fig. 1*d*). No *Nppc* mRNA or CNP was detected in the cerebellum or cartilage from $Nppc^{-/-}$ mice (Fig. 1 *c* and *d*). The *Nppc* transcript was also detected in other tissues, including the cerebrum, pituitary, heart, kidney, ovary, and uterus, in $Nppc^{+/+}$ mice but not in $Nppc^{-/-}$ mice (data not shown).

Analysis of 96 intercrosses between 129/B6 hybrids heterozygous for the disrupted allele revealed that the genotype ratio of +/+:+/−:−/− at weaning (at 4 weeks of age) is 1.00:2.13:0.37 ($n = 648$), indicating a deviation from the expected Mendelian proportions ($P < 0.001$ by χ^2 test). On the other hand, the

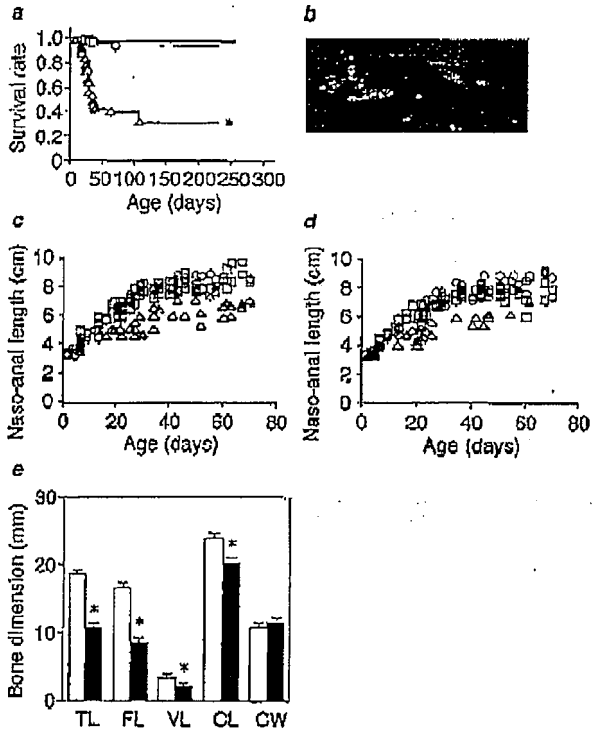

Fig. 2. Dwarfism and mortality in *Nppc*$^{-/-}$ mice. (*a*) Survival curves of *Nppc*$^{+/+}$ (○), *Nppc*$^{+/-}$ (□), and *Nppc*$^{-/-}$ (△) mice. (*b*) Gross appearance of *Nppc*$^{+/+}$ and *Nppc*$^{-/-}$ mice at 10 weeks of age. (*c* and *d*) Growth curves of *Nppc*$^{+/+}$ (○), *Nppc*$^{+/-}$ (□), and *Nppc*$^{-/-}$ (△) mice. (*c*) Males. (*d*) Females. (*e*) Soft x-ray analysis of *Nppc*$^{+/+}$ and *Nppc*$^{-/-}$ mice at 10 weeks of age. Bone dimensions in *e* are of *Nppc*$^{+/+}$ (open bars) and *Nppc*$^{-/-}$ (closed bars) mice ($n = 4$). TL, tibial length; FL, femoral length; VL, fifth lumbar vertebral length; CL, naso-occipital length of the calvarium; CW, maximal interparietal distance of the calvarium. *, $P < 0.05$ vs. *Nppc*$^{+/+}$ mice.

genotype ratio of +/+:+/−:−/− at 16.5 days post coitus was 1.0:1.5:1.4 ($n = 65$), suggesting that *Nppc*$^{-/-}$ mice are not embryonically lethal. More than half of *Nppc*$^{-/-}$ mice died before weaning, and only 30% survived at 100 days of age and thereafter (Fig. 2*a*). No appreciable differences in the genotype ratio and survival rate were noted between the 129/B6 hybrid and 129/Sv pure backgrounds.

At birth, *Nppc*$^{-/-}$ pups had a body weight and a naso-anal length about 90% of those of *Nppc*$^{+/+}$ pups. In *Nppc*$^{-/-}$ mice, dwarfism with short tails and extremities became prominent as they grew (Fig. 2*b*). The naso-anal lengths of male and female *Nppc*$^{-/-}$ mice were 60–70% of those of *Nppc*$^{+/+}$ mice at 4–10 weeks of age (Fig. 2 *c* and *d*). Body weights of *Nppc*$^{-/-}$ mice were about 70% of those of *Nppc*$^{+/+}$ mice, and no significant differences in visceral organ/body weight ratios were noted between genotypes at 20 weeks of age. No other gross abnormalities were found in *Nppc*$^{-/-}$ mice.

Soft x-ray analysis revealed that the longitudinal growth of vertebrae and tail and limb bones is affected in *Nppc*$^{-/-}$ mice. The longitudinal lengths of femurs, tibiae, and vertebrae in *Nppc*$^{-/-}$ mice were 50–80% of those in *Nppc*$^{+/+}$ mice (Fig. 2*e*). The naso-occipital length of the calvarium, which depends on the growth of occipital and sphenoidal bones formed through endochondral ossification, was also reduced significantly in *Nppc*$^{-/-}$ mice relative to *Nppc*$^{+/+}$ mice ($n = 4$, $P < 0.05$) (Fig. 2*e*). On the other hand, in *Nppc*$^{-/-}$ mice, there were no appreciable changes in the shape and interparietal width of the skull vault, which are formed by membranous ossification. These observations indicate that loss of CNP affects endochondral ossification but not membranous ossification *in vivo*.

Longitudinal bone growth is determined by endochondral ossification in the cartilaginous growth plate, which is located at both ends of vertebrae and long bones (22). The cartilaginous growth plate consists of the resting, proliferative, and hypertrophic zones of chondrocytes. Histologically, *Nppc*$^{-/-}$ mice displayed striking narrowing of the growth plate of vertebrae and long bones compared with *Nppc*$^{+/+}$ mice at 7 days of age (Fig. 3 *a* and *b*). The heights of the proliferative and hypertrophic zones were markedly reduced in *Nppc*$^{-/-}$ mice, whereas no significant differences in the resting zone were noted between genotypes. *In situ* hybridization analysis revealed no appreciable difference in the intensity of *Col2a1* and type X collagen (*Col10a1*) mRNA expression between genotypes (Fig. 3 *c–f*). No ectopic expression of *Col1a1* mRNA was found in the growth plate from *Nppc*$^{-/-}$ mice (data not shown). These findings suggest that chondrocyte precursors are capable of differentiating into hypertrophic chondrocytes in the growth plate from *Nppc*$^{-/-}$ mice. However, the band width of *Ihh*-expressing cells [cells committing to differentiation into hypertrophic chondrocytes (23)] was narrowed in *Nppc*$^{-/-}$ mice relative to *Nppc*$^{+/+}$ mice (Fig. 3 *g–j*), although the intensity of *Ihh* expression was not

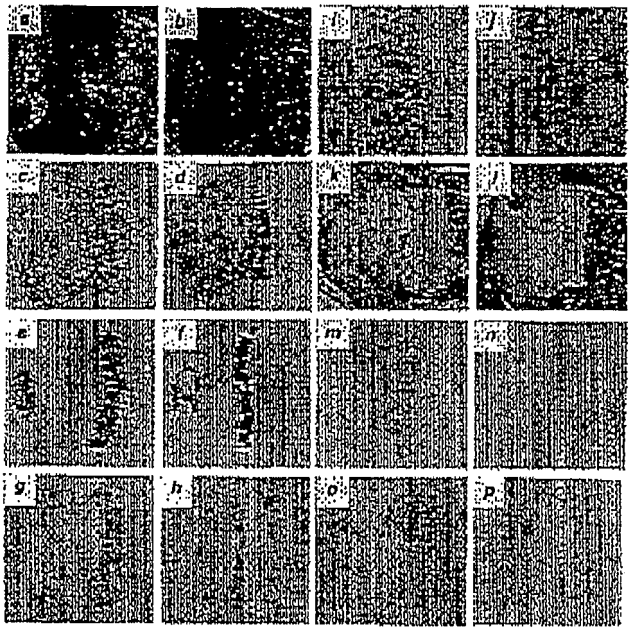

Fig. 3. Hirtological analysis of proximal epiphyseal cartilages of the tibia from 7-day-old *Nppc*$^{+/+}$ and *Nppc*$^{-/-}$ mice. (*a* and *b*) Alcian blue-hematoxylin/eosin staining of tibial epiphyseal cartilages from *Nppc*$^{-/-}$ (*a*) and *Nppc*$^{+/+}$ (*b*) mice. Resting (R), proliferating (P), and hypertrophic (H) zones are indicated. (*c–j*, *m–p*) *In situ* hybridization analysis of tibial epiphyseal cartilages from *Nppc*$^{+/+}$ (*c*, *e*, *g*, *i*, *m*, *o*) and *Nppc*$^{-/-}$ (*d*, *f*, *h*, *j*, *n*, *p*) mice showing expression of mRNA for *Col2a1* (*c*, *d*), *Col10a1* (*e*, *f*), *Ihh* (*g–j*), *Nppc* (*m*, *n*), or *Npr2* (*o*, *p*). (*k* and *l*) Immunohistochemical detection of BrdUrd-labeled chondrocytes in the tibial growth plate from *Nppc*$^{+/+}$ (*k*) and *Nppc*$^{-/-}$ (*l*) mice. (Magnification: *a–h*, *k–p*, ×40; *i*, *j*, ×100.)

remarkably different between genotypes. Notably, the ratio of the height of the hypertrophic zone to the height of the proliferative zone was decreased by ~50% in *Nppc*$^{-/-}$ mice compared with *Nppc*$^{+/+}$ mice (Fig. 3 *a* and *b*). These observations suggest that the rate of cell differentiation into hypertrophic chondrocytes is reduced in *Nppc*$^{-/-}$ mice. The number of hypertrophic chondrocytes positive for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling was not significantly different between genotypes (data not shown), suggesting that chondrocyte apoptosis is unaffected in *Nppc*$^{-/-}$ mice.

We also assessed the consequence of *Nppc* ablation on the proliferation of the growth plate chondrocytes. There was a significant reduction of BrdUrd-labeled cells in *Nppc*$^{-/-}$ mice relative to *Nppc*$^{+/+}$ mice (Fig. 3 *k* and *l*), demonstrating that CNP promotes chondrocyte proliferation *in vivo*.

In *Nppc*$^{+/+}$ mice, *Nppc* mRNA was detected in proliferative and prehypertrophic chondrocytes in the growth plate but not detected in *Nppc*$^{-/-}$ mice (Fig. 3 *m* and *n*). Expression of mRNA for GC-B (*Npr2*) was detected predominantly in the proliferative and prehypertrophic chondrocytes in both *Nppc*$^{-/-}$ and *Nppc*$^{+/+}$ mice (Fig. 3 *o* and *p*). The tail bone cGMP concentrations in *Nppc*$^{-/-}$ mice were markedly smaller than those in *Nppc*$^{+/+}$ mice at 7 days of age (9.7 ± 2.2 vs. 25.6 ± 2.7 pmol/g tissue, $n = 6$, $P < 0.01$), suggesting that CNP is a major determinant of cGMP in the bone. Evidence has indicated that CNP increases the height of the proliferative and hypertrophic zones of chondrocytes and cartilage matrix production in the organ cultures of fetal mouse and rat long bones via cGMP production (21, 24). It is therefore likely that CNP acts primarily on proliferative and prehypertrophic chondrocytes expressing GC-B, thus stimulating growth plate chondrocyte proliferation and differentiation *in vivo* through cGMP-mediated pathway. Histology of other organs was not remarkable in *Nppc*$^{-/-}$ mice at 20 weeks of age (data not shown).

Transgenic Rescue of Nppc$^{-/-}$ Mice. To determine whether local expression of CNP in the bone can rescue the dwarfism of *Nppc*$^{-/-}$ mice *in vivo*, they were crossed with mice with transgenic expression of CNP in the growth plate chondrocytes (Tg mice). The genetically "rescued" animals [or *Nppc*$^{-/-}$ mice with the transgene expression (Tg/*Nppc*$^{-/-}$ mice)] were of normal appearance (Fig. 4*a*), and their skeletons were indistinguishable from those of *Nppc*$^{+/+}$ mice. During postnatal development, no significant difference in the naso-anal length was observed between Tg/*Nppc*$^{-/-}$ and *Nppc*$^{+/+}$ mice (Fig. 4*b*). Body weights of Tg/*Nppc*$^{-/-}$ mice were not different from those of *Nppc*$^{+/+}$ mice at 10 weeks of age (20.3 ± 1.9 vs. 21.9 ± 1.1 g, $n = 6$). Histologically, no appreciable differences in tibial growth plate cartilage were noted between Tg/*Nppc*$^{-/-}$ and *Nppc*$^{+/+}$ mice (Fig. 4 *c–e*). The tail bone cGMP concentrations in Tg/*Nppc*$^{-/-}$ mice (17.2 ± 4.9 pmol/g tissue, $n = 6$) were also roughly comparable to those in *Nppc*$^{+/+}$ mice (25.6 ± 2.7 pmol/g tissue, $n = 6$, $P > 0.05$). These findings demonstrate that CNP, when expressed locally in the growth plate chondrocytes, can rescue the skeletal defect of *Nppc*$^{-/-}$ mice via the cGMP-mediated mechanism *in vivo*. Although CNP is expressed in a variety of central and peripheral tissues, the data of this study indicate that CNP acts locally as a positive regulator of endochondral ossification. Targeted expression of CNP in the growth plate chondrocytes also resulted in prolonged survival of *Nppc*$^{-/-}$ mice; most Tg/*Nppc*$^{-/-}$ mice examined (11 of 13 mice) survived up to

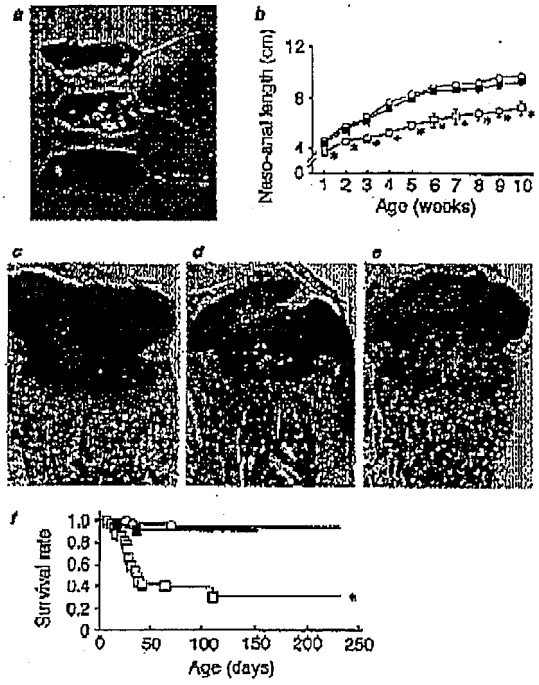

Fig. 4. Genetic rescue of *Nppc*$^{-/-}$ mice by crosses with transgenic mice with targeted expression of CNP in the growth plate chondrocytes. (*a*) Gross appearance of *Nppc*$^{+/+}$ mice, *Nppc*$^{-/-}$ mice without the transgene expression, and *Nppc*$^{-/-}$ mice with the transgene expression (Tg/*Nppc*$^{-/-}$ mice) at 20 weeks of age. (*From Top to Bottom*) *Nppc*$^{+/+}$, *Nppc*$^{-/-}$, and Tg/*Nppc*$^{-/-}$ mice. (*b*) Growth curves of *Nppc*$^{+/+}$ (○), *Nppc*$^{-/-}$ (□), and Tg/*Nppc*$^{-/-}$ (■) mice. *$P < 0.05$ vs. *Nppc*$^{+/+}$ mice. (*c–e*) Alcian blue–hematoxylin/eosin stainings of tibial epiphyseal cartilages from 7-day-old *Nppc*$^{+/+}$ (*c*), *Nppc*$^{-/-}$ (*d*), and Tg/*Nppc*$^{-/-}$ (*e*) mice. (*f*) Survival curves of *Nppc*$^{+/+}$ (○), *Nppc*$^{-/-}$ (□), and Tg/*Nppc*$^{-/-}$ mice (■). *, $P < 0.05$ vs. *Nppc*$^{+/+}$ mice.

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20 weeks of age (Fig. 4*f*). Therefore, *Nppc*$^{-/-}$ mice are short-lived because of their skeletal abnormalities.

Transgenic mice with elevated plasma BNP concentrations show skeletal overgrowth due to increased endochondral ossification (13, 14), whereas mice with targeted disruption of BNP exhibit no skeletal abnormalities (18). Thus, BNP, a hormone derived from the cardiac ventricle, is not involved in endochondral ossification under physiological conditions. This study has established CNP as an endogenous ligand to activate the cGMP production in the bone, thereby regulating endochondral ossification. In this context, mice with targeted disruption of cGMP-dependent protein kinase II, an intracellular mediator of cGMP that is expressed in late proliferative and early hypertrophic chondrocytes in the growth plate, show dwarfism due to impaired endochondral ossification (25). We postulate that the CNP/GC-B/cGMP-dependent protein kinase II signaling cascade plays a critical role in endochondral ossification. Recently, *Npr3*$^{-/-}$ mice have been reported to show skeletal overgrowth and increased endochondral ossification (15, 16). It is likely that the metabolic clearance of CNP produced locally is delayed in *Npr3*$^{-/-}$ mice, thereby activating endochondral ossification.

The *Nppc*$^{-/-}$ mice exhibit gross phenotypes and histologic features similar to those found in patients with achondroplasia (26) and mouse models for achondroplasia (27, 28). Several gain-of-function mutations in the fibroblast growth factor receptor 3 gene have been found in most achondroplastic patients and those with two other distinct skeletal dysplasias, hypochondroplasia and thanatophoric dysplasia (29–32). Our data suggest that CNP may be one of the causative genes for such skeletal dysplasias of unknown origin and may be useful for treatment of achondroplasia.

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文献 2

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GENE AND PRECURSOR STRUCTURES OF HUMAN C-TYPE NATRIURETIC PEPTIDE

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SUMMARY: We have isolated the gene for human C-type natriuretic peptide (CNP) from a human genomic library using a cloned porcine CNP genomic DNA fragment as probe. Human CNP gene consists of at least two coding blocks and an intron, and encodes a 126-residue CNP precursor (human prepro-CNP). From a comparison of the amino acid sequences of porcine and rat prepro-CNPs, human prepro-CNP is found to be processed to generate 22-and 53-residue peptides (human CNP-22 and human CNP-53, respectively) as major endogenous CNPs in human. Interestingly, human CNP-53 has two amino acid substitutions as compared to the porcine and rat CNP-53s, whereas human CNP-22 is identical to the porcine and rat CNP-22s. Intravenous injection of human CNP-53 into anesthetized rats induces diuretic-natriuretic and hypotensive activities with same potencies as porcine CNP-53 does, although these activities were considerably lower (about 1/100) than those of human α-ANP.

C-type natriuretic peptide (CNP) is the third member of the natriuretic peptide family identified following atrial natriuretic peptide (A-type natriuretic peptide, ANP) (1) and brain natriuretic peptide (B-type natriuretic peptide, BNP) (2). From porcine brain, two related CNPs, a 22-residue peptide (porcine CNP-22) and its N-terminally elongated peptide (porcine CNP-53; 53-residue), have been identified and found to be major molecular forms of CNP in the brain (3, 4). CNP has considerable sequence similarity to ANP and BNP within the 17-residue ring structure formed by a disulfide linkage. Unlike ANP and BNP, however, CNP ends at the second cysteine residue and lacks further C-terminal extension from the ring structure. CNP also exerts a pharmacological spectrum similar to those of ANP and BNP, including diuretic-natriuretic, hypotensive and chick rectum relaxant effects, although the relative potencies are different (3).

Abbreviations: CNP, C-type natriuretic peptide ; ANP, atrial natriuretic peptide (A-type natriuretic peptide); BNP, brain natriuretic peptide (B-type natriuretic peptide); HPLC, high performance liquid chromatography.

0006-291X/91 $1.50

Thus, three types of natriuretic peptides are now thought to participate in regulating the homeostatic balance of body fluid volume and blood pressure in mammals.

Knowledge of the precursor and gene structures for CNP would be helpful in understanding the physiological function of CNP and its functional differences from the other two types of natriuretic peptides (ANP and BNP). In this respect, we have recently defined the precursor structures of porcine (5) and rat CNPs (6) by molecular clonings of their corresponding gene and cDNA, respectively.

Here we describe the isolation and characterization of the gene for human CNP. The entire precursor structure and predicted endogenous forms for human CNP will be also presented.

Materials and Methods

Isolation of genomic clones (λhCNP2 and λhCNP1): The 150 base pair (bp) porcine genomic DNA fragment encoding porcine CNP-53 (5) was used as first probe to screen a Charon 4A human genomic library made from human liver DNA. From one positive clone (λhCNP2) isolated from 2×10^5 phage clones, a 2.0-Kb *EcoRI* restriction fragment that hybridized to the probe was isolated and sequenced. Then, this 2.0-Kb *EcoRI* fragment was used as second probe to rescreen the library, and λhCNP1 was isolated. Southern blot analysis defined that λhCNP1 possessed a 4.0-Kb *EcoRI* restriction fragment that hybridized to the 2.0-Kb *EcoRI* fragment. This fragment was isolated and used for further analyses.

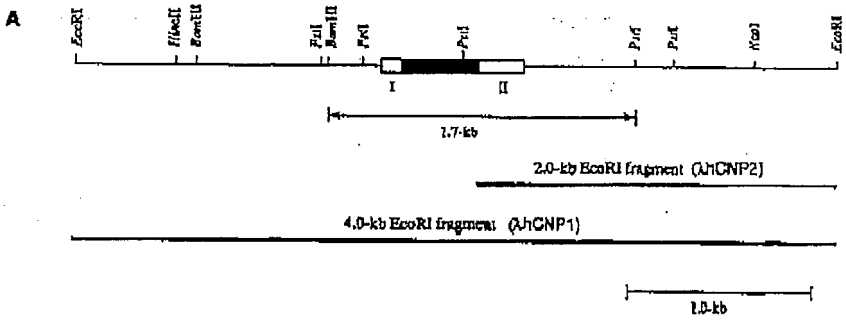
Nucleotide sequence determination: The 2.0-Kb and 4.0-Kb *EcoRI* restriction fragments obtained above were subcloned into pUC19 vector, respectively, and restriction enzyme maps of the 2.0-Kb and 4.0-Kb *EcoRI* fragments were made. Appropriate restriction fragments were subcloned into M13mp18 or M13mp19 to generate single strand templates, and sequenced both strands by chain termination method (7) using synthetic oligonucleotide primers and universal primers.

Peptide synthesis: Human CNP-53 and porcine CNP-53 were prepared by solid phase techniques conducted on a phenylacetamidomethyl resin using a peptide synthesizer (Applied Biosystems 430A). An intramolecular disulfide linkage was formed by the action of $K_3[Fe(CN)_6]$. Synthetic peptides were purified by ion exchange HPLC and reverse phase HPLC, and correct synthesis was confirmed by amino acid analysis and sequencing.

Bioassays: Natriuretic and diuretic activities were assayed as described previously (8), after injection of peptides into assay rats through the femoral vein in one shot. Systemic blood pressure was measured from the carotid artery in rats(8).

Results and Discussion

From a human genomic library, two phage clones (λhCNP2 and λhCNP1) containing human CNP gene sequences were isolated as described in Materials and Methods. The clone isolated first (λhCNP2) contained a 15.5-kilobase (Kb) insert, in which a 2.0-Kb *EcoRI* restriction fragment had an open reading frame that encoded a protein (88-residue) highly homologous to the C-terminal region of the precursor for porcine CNP. This open reading frame, however, started from the *EcoRI* site that is located at the end of the insert and didn't contain a likely site of translational initiation, indicating that λhCNP2 was a partial clone lacking nucleotides encoding N-terminal regions of a precursor for human CNP. The clone isolated second (λhCNP1) contained a 4.0-Kb *EcoRI* restriction fragment that hybridized to the 2.0-Kb fragment of λhCNP2. As shown in Fig.1A, the 4.0-Kb *EcoRI* fragment possessed about 2.0-Kb nucleotide extending beyond the 5' end of the 2.0-Kb *EcoRI* fragment.



B

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CGATCGGTCGGGGGTGGGATAAGGGAGGGGAGGCCCCGCGGCCCCGCTGGCGGCCGTCGGCGCGGCCGCGTGCGTCGTGTC   80
ATTGTCCCGGGGGGCCCCCTGCCCCGAGGATCACATCAGCGGCAGGTTGCATTAAAAGCCGCGAGCAGAGTCACGGGC   160
Y box   GC box      GC box                          TATA box
TCAGAGCGCACGCAGCCGGCGCCGCGCAGCACTGGGACCGCTGCTCGCCCTGCAGCGCAGCGCAGGCTGCTGGGGATCCCCC   240
TGGTGGTCTGCCGGCGCAACCTGCGCGCCCTCGCTGCCGCCCGTGTGCGCCCCTTGACCCCAACCGCCACCATGCATCTTTC   320
                                                                    MetHisLeuSe
                                                                    1
CCAGCTGCTGCTCCTGGCCCCTGCTGCTCAGCCTCGTCTCCCTGGGCGGCTGGGAAGCCAAGCCCGGGGCGCCGCCGAAGG   400
rGlnLeuLeuAlaCysAlaLeuLeuLeuThrLeuLeuSerLeuArgProSerGluAlaLysProGlyAlaProProLys
        -       10                              20                              30
TGGGTGCTGTCGTGGGGACAGCCGCAGCCTGGGAGAGGCGTGGCAGGCTGGGCGCTTGCAGAATGCCGCGCCGCAGGACCCAG   480
GAGACAGGGAAGGCAGGCGCGCTGTCTCCTCCGAGATGCGCCTCGGCCACAGCCCGGCAGCCCTGCAAGCGCGATTCCGG   560
GGTCGACTTCTCCACCCTCCGGAGAAACTGGCCCCATGCGCAGCCCCCTACCCCAGTGTGGCCTGCCCCCCCACAGCAA   640
AGGCAGGGCAGGGGGCTTCCGGAGGGAGCGGGAAGGCGGCCGCGTGGCAGGTGGATGCGGGGCCAAGCTGGCCGGCATC   720
GGTGGGGCGGGCTCTGGGCTTGGGAGGGACAGCGCGGCGCGGCGGGCGCGTGGGGGTGGAGCATCAGAGTCCCCCTGCT   800
GCAGCCGCCTGTCCGTTCACCTGCCGCGCTGTTTCGTCGCACAGGTCCCCGGAACGCCCCCGCAGAGGAGCTGGCCGAGC   880
                                        ValProArgThrProProAlaGluGluLeuAlaGLuP
                                        31                              40
CGCAGGCTGGGGGCGGCGGCTCAGAAGAAGGGCCACAAGGCTCCCGGGGGCGCGCCCCCCCCAATCTCAAGGGCGCAGCGCTCG   960
roGlnAlaAlaGlyGlyGlyGlnLysLysGlyAspLysAlaProGlyGlyGlyGlyAlaAsnLeuLysGlyAspArgSer
                50                              60
CGACTGGTCGCGCACCTGCGCGTGGACAGCAAGTCGCGGGCAGCGTGGGCTCGGCTTCTGCAAGAGCACCCCAACGCCCG  1040
ArgLeuLeuArgAspLeuArgValAspThrLysSerArgAlaAlaTrpAlaArgLeuLeuGlnGluHisProAsnAlaAr
 70                              80                              90
CAAATACAAAGGAGCCAACAAAAAGGCCTTGTCCAAACCGTCCTTCGGCCTCAAGGTCGCACCGAATCGGCTCCATGAGCG  1120
gLysTyrLysGlyAlaAsnLysLysGlyLeuSerLysGlyCysPheGlyLeuLysLeuAspArgIleGlySerMetSerG
        100                             110                             120
GCCTGGGATGTTAGTGCCGCCCCCCCCTGGCGGCGGTGAGTACGGCCGCAGCGGAGCCCAGCCCCAGCCCGGCCCGGTACC  1200
lyLeuGlyCys***
        126
GCCCGCCCCCGCAGCCAGCCTTCCGAACCGCCCGCAGCCGCCTTTGCTCAAGTTGTGCTAGCCGTTTGCCAGCCGGCCCGTT  1280
TATTATCCCACTTTACAGACAAACAAGCGAAGCATAACGTCATCGGGGAAGTTTGGCAAGGTCAGAAACGGGTCAGCGT  1360
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TACAGCCACTGGCCCCTATTGGCCAGCTGGTGGGGGGCATTGAGAGGAGATCATGGGTTTGTGGGAGCAGAAGAACGAAGGT  1520
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CTGAGAGCCCCGTTGTGCCTGGGACAGTCCGATTCGTGTCGCTTCTCTAAACCGAGGCAGTGGCAAACTGGTCTGTCCA  1680
GCGTCGTGAGCCAGCTGCAG 1700
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Figure 1. (A) Restriction maps for the 2.0-Kb and 4.0-Kb *Eco*RI fragments carried on λhCNP2 and λCNP1, respectively. Only those restriction sites relevant to the present work are shown. The coding block and intron of human CNP gene were shown by ▭ and ■, respectively. ↔ shows the 1.7-Kb *Bam*HI-*Pst*I restriction fragment whose nucleotide sequence shown below.
(B) Nucleotide and deduced amino acid sequences of the 1.7-Kb *BamHI-PstI* restriction fragment. Nucleotide residues are numbered from the *BamHI* site. The deduced amino acid sequence, shown below the nucleotide sequence, is numbered from the initiation methionine (Met). Putative TATA box, GC box and Y box are indicated by shaded boxes. The translational stop codon is shown by ***.

Fig.1B shows nucleotide sequence of the 1.7-Kb *Bam*HI-*Pst*I restriction fragment in the 4.0-Kb *Eco*RI fragment (Fig.1A) and deduced amino acid sequence. This fragment possesses the entire coding region for human CNP precursor protein as well as a promoter like region.

About 135 bases downstream of the 5' terminal *Bam*HI site, there is a TATAA sequence (TATA box) which is homologs to the consensus sequence for RNA transcription in eukaryotic genes (9). In the 5' flanking region of the TATA box, two GC boxes (10) and a potential Y box (11) are found.

The human CNP gene consists of at least two coding blocks and an intron (Fig.1). Coding block I and coding block II encode 30 and 96 amino acid residues, respectively. The intron localized between the two coding blocks is 444-bp long, and interrupts the reading frame precisely between codons and contains the GT and AG consensus dinucleotides (12) at the 5' and 3' boundaries, respectively. This structural organization is almost identical to that of porcine counterpart, i.e., the sizes of each coding blocks (I and II) and intron are identical in both species, except that the intron of human CNP gene is 5-bp longer than that of porcine gene, and the similarity of the human and porcine nucleotide sequence in each coding blocks (I and II) and intron are 94, 93 and 78%, respectively.

The human CNP precursor (126-residues) encoded in the two separate coding blocks exhibits a typical prepro-structural form. The N-terminal 23 amino acid peptide (Met 1 to Ala 23) exhibits characteristic features for the signal peptides generally found in prosecretory proteins (13). Therefore, it is likely that the first processing of human CNP precursor (human prepro-CNP) takes place at the Ala 23- Lys 24 bond to generate the 103-residue pro-CNP. The 22 amino acid peptide at the carboxyl end of the pro-CNP (Gly 105 to Cys 126) is identical to the porcine CNP-22, which is one of the endogenous CNPs identified in porcine brain(3), and this peptide is preceded by a typical processing signal (Lys 103-Lys 104). These facts suggest that further processing takes place after Lys 104 to generate the 22-residue peptide, and this peptide, designated human CNP-22, can be regarded as an endogenous CNP in the human. Furthermore, human pro-CNP has the arginine residue at position 73 as porcine precursor does (Fig.2A), and it has been defined that the porcine pro-CNP was processed after this arginine residue to give another endogenous CNP (porcine CNP-53)(4,5). Consequently, it is likely that the C-terminal 53 amino acid peptide (Asp 74 to Cys 126) of human pro-CNP is also generated from the precursor as another endogenous CNP (human CNP-53).

Fig.2A summarizes the amino acid sequence data of prepro-CNPs of three mammalian species reported in this (human) and earlier papers (porcine and rat) (5,6). We previously pointed out that CNP seemed to be the most conserved among the three natriuretic peptide families (6). The present identification of human CNP precursor has confirmed it, i.e., human prepro-CNP has only 5 and 8 amino acid substitutions as compared to the porcine and rat counterparts, respectively. However, it should be noted that the human CNP-53 has two amino acid substitutions at the 26th and 37th positions from the C-terminus as compared to the porcine and rat CNP-53s, whereas the human CNP-22 is identical to the porcine and rat CNP-22s.

Since the human CNP-53 thus deduced has two amino acid substitutions, we synthesized the human CNP-53 along with the porcine CNP-53, and measured their diuretic-natriuretic and hypotensive activities as described in Materials and Methods. As shown in Table 1, intravenous

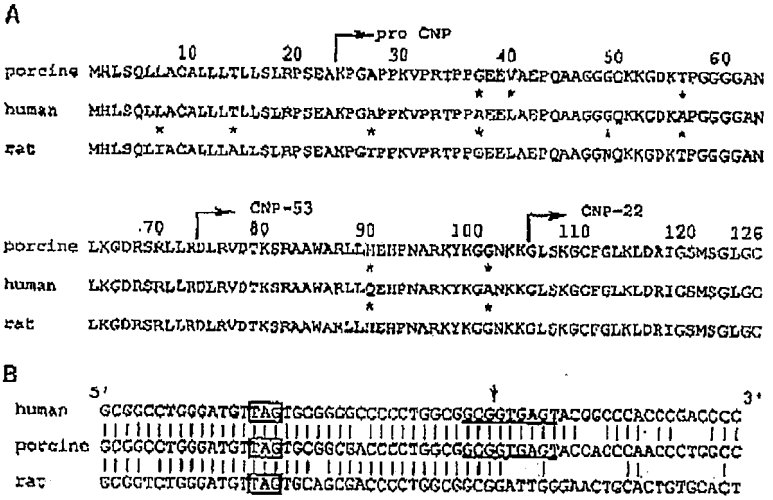

Figure 2. (A) Comparison in amino acid sequence of human, porcine and rat prepro-CNPs. One-letter amino acid notation is used. Positions for amino acid substitution observed between human and porcine, and human and rat are indicated by *.
(B) Nucleotide sequence similarity in the 3'-flanking regions of human and porcine CNP genes and rat CNP cDNA. Box shows the translational stop codon. The likely sequences for consensus splicing donor sequences are shown by underling with a putative splicing site↓.

injection of the human and porcine CNP-53s into anesthetized rats induced diuretic-natriuretic and hypotensive activities essentially in the same manner as those of human α-ANP, although these activities of human and porcine CNP-53s were, as like CNP-22 (3), considerably lower (about 1/100) than those of human α-ANP.

In previous paper (6), we showed that rat CNP gene was expressed exclusively in brain but not in other tissues including cardiac atrium, ventricle, lung, kidney, liver, and gastrointestinal tract. On the other hand, rat ANP gene was mainly expressed in atrium and also expressed in

Table 1. Diuretic and hypotensive activities of human and porcine CNP-53s

peptide	dose (nmole/kg)	Urine (%)	Na^+ (%)	K^+ (%)	Cl^- (%)	Blood Pressure (%)
α-hANP	1.0	265±22	318±51	176± 5	291±28	88.7±1.7
human CNP-53	100	250±31	335±74	198±34	286±65	90.2±1.6
porcine CNP-53	100	235±36	296±32	201±30	259±31	90.8±1.1

Mean±S.E. from 4 rats.
% changes before and after administration.

ventricle and brain. In the case of rat BNP, the gene expression was found in atrium and ventricle but not in brain. In this respect, both human and porcine CNP genes possesses two GC boxes and a potential Y box in their promoter regions but such transcriptional regulatory sequences are not found in the promoter regions of ANP and BNP genes(14-19), suggesting that these sequences may be responsible for the tissue specificity and the level of gene expression of CNP.

In human CNP gene, the 3' flanking region downstream of the termination codon (nucleotide position 1132 to 1134 in Fig.1B) does not contain an AATAAA sequence, which is known as a polyadenylation signal in many eukaryotic mRNA (20). Further sequencing of about 1.3-Kb downstream of the 3' terminal *Pst*I site failed to show the existence of this sequence. This suggests that the human CNP gene has a very long 3' untranslated region and/or there is a 2nd, long intron in this region. To clarify this, we compared the nucleotide sequences of the 3' flanking region of human CNP with those of the porcine CNP gene and rat CNP cDNA. As shown in Fig.2B, the nucleotide sequences of the human, porcine and rat are conserved down to about 20 nucleotides downstream of the termination codons, beyond which the similarity declines rapidly, especially between the genomic sequences and the cDNA sequence. Moreover, the human and porcine CNP genes contain likely sequences for the consensus splicing donor sequences(21,22), C/AAGGTA/GAGT, around 20-bp downstream of the termination codon. These data suggest that the human and porcine CNP genes seem to contain the second intron in the 3' flanking region.

The human CNP genomic DNA isolated here will provide the means to identify the tissues that express the gene and to study the control mechanisms of human CNP gene expression. The investigation on these issues will help to understand the physiological function of CNP and its functional differences from the other two types of natriuretic peptide, ANP and BNP. Furthermore, human precursor structure as well as endogenous forms for human CNPs thus deduced will facilitate identification of endogenous CNP forms in *vivo*.

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Cloning and sequence analysis of a cDNA encoding a precursor for rat C-type natriuretic peptide (CNP)

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Recent identification of a C-type natriuretic peptide (CNP) in porcine brain strongly suggested that a third member of the natriuretic peptide family still remains to be identified in other species of mammals. A cDNA encoding a precursor for rat CNP was cloned from a rat brain cDNA library and sequenced. The precursor was a 126-residue peptide, carrying a 23-residue signal sequence at the N-terminus and the known porcine CNP-53 sequence at the C-terminus. By RNA blot analysis, rat CNP mRNA was found to be expressed exclusively in the brain, implying that CNP may function in the central nervous system as a neuropeptide.

C-type natriuretic peptide; cDNA cloning; Precursor structure; A-type natriuretic peptide; B-type natriuretic peptide; Neuropeptide

1. INTRODUCTION

Identification of atrial natriuretic peptide (A-type natriuretic peptide: ANP) and brain natriuretic peptide (B-type natriuretic peptide: BNP) disclosed the possibility that a natriuretic peptide (NP) family comprising similar hormones participates in regulating the homeostatic balance of the body fluid volume and blood pressure [1-3]. ANP and BNP, though they are derived from distinct genes, share a highly homologous 17-residue ring structure, which is known to be essential for exerting natriuretic/diuretic and hypotensive activities. We have recently identified in porcine brain a third member of the NP family and designated it C-type natriuretic peptide (CNP) [4,5]. Porcine CNP consisting of 22 amino acid residues (CNP-22) and its N-terminally elongated form with 53 residues (CNP-53) show remarkable sequence homology to ANP and BNP within the 17-residue ring portion formed by a pair of cysteine residues. However, porcine CNP terminates at the second cysteine residue, which participates in the ring formation, and completely lacks the further C-terminal extension, which occurs in both ANP and BNP. Moreover, porcine CNP-22 stimulates guanylate cyclase activity in cultured vascular smooth muscle cells more potently than ANP and BNP, implying that CNP may function in a manner distinct from ANP and BNP [6]. In order to characterize the specified physiological role of CNP and to define its functional difference from ANP and BNP, it is essential to know the structure of rat CNP, since extensive studies on ANP and BNP have been carried out mainly with peptides of rat and human origin. Here we report cloning and sequence analysis of a cDNA encoding a precursor for rat CNP. In addition, regional difference in the expression of the CNP gene was also examined by RNA blot analysis.

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The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number no. D90219

2. MATERIALS AND METHODS

2.1 *cDNA library construction*

Total RNA was extracted from rat brain by the guanidine thiocyanate method. Poly(A)$^+$ RNA was isolated on an oligo(dT)-cellulose column (Pharmacia). Double-stranded cDNA was synthesized from 4 μg of rat brain poly(A)$^+$ RNA by the method of Gubler and Hoffman [7]. cDNA was ligated to *Eco*RI adaptors and size-fractionated by 1% agarose gel electrophoresis. After electroelution, 300-1500 bp-fraction was ligated to phage λgt10 arms (Bethesda Research Laboratory) and packaged in vitro by using Gigapack Gold (Stratagene).

2.2 *cDNA library screening and sequence analysis*

High- and low-stringencies in hybridization were controlled by the two different concentrations of formamide (50% and 20%). Hybridization was performed at 37°C in a solution of 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, containing 50% or 20% formamide. Rat CNP genomic clones were obtained from Charon4A rat genomic library (Clonetech). Recombinants from the library were screened under low-stringency conditions, by using a 150-bp genomic DNA fragment encoding porcine CNP-53 as a probe, which was kindly donated by Y. Tawaragi (Suntory Institute for Biomedical Research). As reported [8], the probe was prepared from porcine liver DNA by the PCR-amplified method, using sense and antisense primers, corresponding to the N-terminal and C-terminal sequences of porcine CNP-53. One of the clones thus isolated was partially sequenced by the dideoxy chain termination method. Then, the *Sma*I/*Mva*I fragment of the clone, corresponding to nucleotides 245-394 in Fig. 1, was used as a

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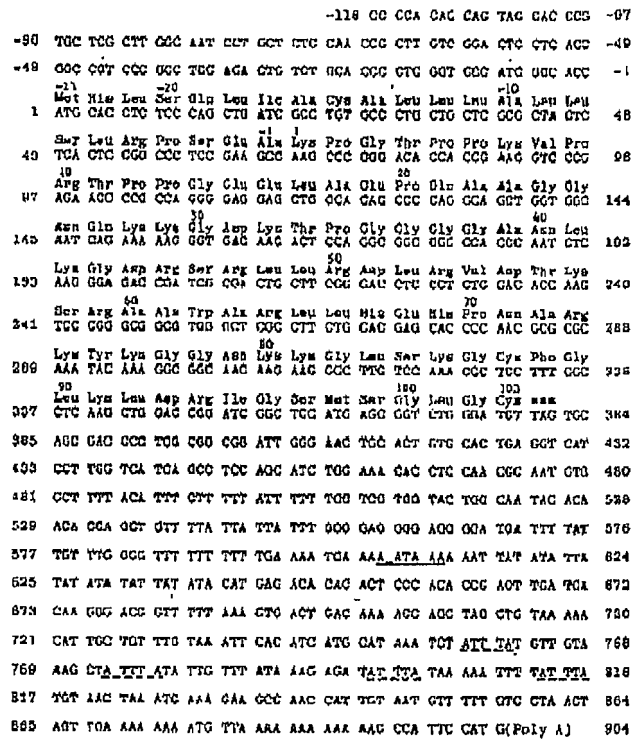

Fig. 1. Nucleotide sequence of the cDNA insert in λrCNP21 with predicted amino acid residues. Nucleotide residues are numbered beginning with the first residue of ATG triplet encoding a putative initiating methionine, and those on the 5'-side of nucleotide 1 are indicated by negative numbers. The termination codon is marked with three consecutive asterisks. The AATAAA sequence is underlined. Dashed lines under the 3'-untranslated region indicate ATTTA motifs.

probe to screen the rat brain cDNA library. Approximately 6×10^6 recombinant phages were transferred to nitrocellulose membranes and screened under high-stringency conditions. A positive clone λrCNP21 harboring the longest cDNA insert was subcloned into M13 vector and sequenced. Both strands of the cDNA clone were sequenced.

2.3 *RNA blot analysis*

Poly(A)$^+$ RNA (20 μg) was denatured using glyoxal and dimethylsulfoxide, and was fractionated on a 1.4% agarose gel. After electrophoresis, RNA was transferred to a nylon membrane (Zeta Probe, Bio-Rad) and fixed by ultraviolet irradiation (Stratalinker, Stratagene). The membrane was prehybridized and hybridized at 37°C in 50% formamide, 6 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. A cDNA insert of λrCNP21 was labeled by the random-primed method and used for the hybridization. The blot was washed at 37°C once in 3 × SSC, 0.1% SDS, once in 0.5 × SSC, 0.1% SDS, and finally twice in 0.1 × SSC, 0.1% SDS. After autoradiography, the radiolabeled probe was removed by boiling the membrane in 0.1 × SSC, 0.5% SDS for 20 min. Then, the membrane was used for the hybridization by the ANP or BNP probe.

3. RESULTS AND DISCUSSION

Recombinants obtained from a rat genomic library were first screened under low-stringency conditions by using a 150-bp porcine CNP probe corresponding to porcine CNP-53 [5,8]. Five positive clones thus obtained exhibited the identical restriction inserts of about 19 kbp long. One of the clones obtained above was partially sequenced to verify that the clone encoded the rat CNP gene. Then, the *SmaI/MvaI* fragment of the clone corresponding to the CNP-53 region was used as a pro-

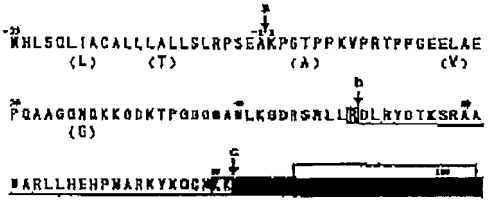

Fig. 2. Amino acid sequence of rat prepro-CNP. Amino acid replacements observed between rat and porcine CNP precursors are shown in the parentheses. One-letter amino acid notation is used. Arrows indicate the putative processing sites for the signal peptidase (a), and for generating CNP-53 (b) and CNP-22 (c). Processing signals for CNP-53 and CNP-22 are boxed. CNP-53 portion is underlined; CNP-22 portion is shaded.

be for screening the rat brain cDNA library under high-stringency conditions.

Among 22 positive clones obtained from 6×10^6 recombinant phages, a clone, λrCNP21, harboring the longest cDNA insert, was sequenced. Fig. 1 shows the complete nucleotide sequence of the cDNA, which is 1020 bp long (excluding poly(A) tail). A putative initiation codon ATG is located at nucleotides 1-3, preceded by the consensus sequence for the initiation, while a termination codon TAG is found 126 codons later at nucleotides 379-381. A typical polyadenylation signal, AATAAA, is found only at nucleotides 606-611, but no poly(A) tail follows the signal. A GTTAAA sequence, the closest match to the signal, which is located 26 nucleotides upstream of the polyadenylation site, may serve as a signal for the polyadenylation.

The amino acid sequence encoded in the open reading frame is deduced as shown in Figs 1 and 2. The amino acid sequence flanked by Cys^{87} and Cys^{103} (nucleotides 328-378) corresponds to the 17-residue ring structure characteristic of the NP family. In the cDNA sequence, a TGT codon for the C-terminal Cys^{103} is directly followed by a termination codon. Thus, the open reading frame encodes a putative precursor for rat CNP (rat prepro-CNP), which consists of 126 amino acid residues and carries a bioactive unit at its C-terminus.

The first 23-residue peptide starting from the initial methionine is thought to be a signal peptide, based on its characteristic hydrophobic features. Consequently, it is most likely that the first processing of the precursor takes place in between Ala^{-1} and Lys^{1} to generate a 103-residue pro-CNP (Fig. 2). In the rat prepro-CNP, a typical processing signal, Lys^{38}-Lys^{61}, is followed by a 22-residue C-terminal peptide, which is identical to porcine CNP-22, one of the endogenous CNPs in porcine brain [4]. This fact strongly suggests that the C-terminal 22-residue peptide (rat CNP-22) can also be regarded as an endogenous CNP in the rat. Moreover, the C-

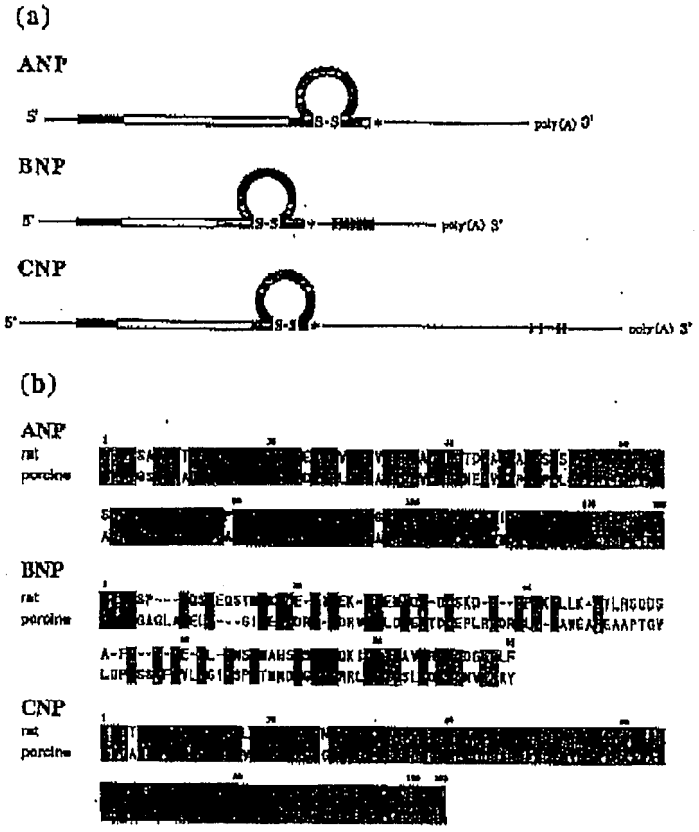

Fig. 3. (a) Schematic diagram of cDNAs for rat natriuretic peptide family. ▨ signal peptide; ▬ bioactive unit; S-S disulfide bond; * stop codon; ▪ ATTTA motif. (b) Comparison in amino acid sequence of rat and porcine natriuretic peptide families. Sets of identical residues are shaded. References of the sequences; rat ANP [9], porcine ANP [13], rat BNP [10], porcine BNP [14], porcine CNP [8].

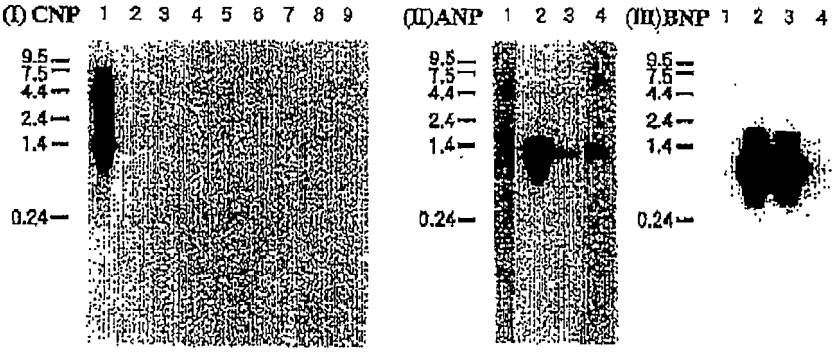

Fig. 4. RNA blot analysis of rat natriuretic peptide transcripts in rat tissues. Each lane contained 20 μg of poly(A)$^+$ RNA. Numbers on the left indicate kilobase as determined from RNA size markers. The probes used for hybridization are (I) CNP probe, (II) ANP probe and (III) BNP probe. Lanes: (1) brain; (2) atrium; (3) ventricle; (4) lung; (5) liver; (6) kidney; (7) stomach; (8) small intestine; (9) large intestine. Autography was at −80°C for 5 days except for (II)–2 and –3 (−80°C for 2 h).

terminal 53-residue sequence (rat CNP-53) is preceded by a single Arg^{50}, as in the case of the porcine CNP precursor (Fig. 2). Thus, it its likely that the processing may take place after Arg^{50} to give another endogenous form (CNP-53).

As reported in our previous papers, porcine CNP-22 as well as CNP-53, unlike ANP and BNP, have the unique structural feature that the C-terminal extension from the ring structure is completely absent [4,5]. The present analysis shows that the codon for the C-terminal Cys^{103} is directly followed by a termination codon. This fact clearly indicates that rat CNP also lacks the C-terminal extension and that this form is generated without any additional post-translational modification.

We have recently accomplished the structural analysis of the porcine CNP gene and deduced the amino acid sequence of a porcine CNP precursor (porcine prepro-CNP) [8]. Surprisingly, rat prepro-CNP is identical to porcine prepro-CNP, except that only 5 amino acid residues are replaced (Fig. 2).

As schematically indicated in Fig. 3a, the molecular construction of prepro-CNP is very similar to those of ANP and BNP. All members of the rat NP family carry the signal sequences at their N-terminal regions and the bioactive units at their C-terminal regions, although they are derived from distinct genes [9,10]. However, the 3′-untranslated region of the rat CNP cDNA is about two times longer than those of rat ANP and BNP cDNAs. Furthermore, the rat CNP cDNA contains in the 3′-untranslated region four copies of an ATTTA motif (nucleotides 757–761, 774–778, 797–801 and 812–816), which is known to destabilize mRNA in the cell [11]. Since the motif is very much clustered in BNP, but is not found at all in ANP, the expression of the three genes for the natriuretic peptide family may be regulated through the different mechanisms [10]. It is well known that nucleotide and amino acid sequences of ANP precursors are highly conserved among various mammals [9,12] (Fig. 3b). In contrast with this, sequence homology among BNP precursors is remarkably low and homologous sequences are limited in the signal peptide and the C-terminal bioactive unit [10]. However, homology between rat and porcine CNP precursors is remarkably high (97.1% in the amino acid sequence of the pro-form), much higher than that between ANP precursors (86.7%). Thus, mammalian CNP is thought to be the most conserved NP in the family. In this respect, it should be mentioned that the C-terminal 22-residue peptide of a human CNP precursor has very recently been established to be identical to those of rat and porcine CNP-22 (to be published).

Fig. 4 shows the RNA blot analyses of the rat NP family. When a cDNA insert of λrCNP21 was used as a CNP probe, mRNA of approximately 1200 bp, which is longer than those of rat ANP (950 bp) and rat BNP (850 bp), was detected only in brain, and not in other examined tissues including cardiac atrium and ventricle. Furthermore, no positive plaque was obtained, when a rat atrial cDNA library was screened with the CNP probe. Thus, rat CNP is concluded to be expressed exclusively in brain but not in atrium and ventricle, while rat ANP and BNP are mainly localized in heart. ANP and BNP, although their expression is regulated in their own manner, are thought to be cardiac hormones mainly secreted from heart, while CNP is expressed only in brain and presumably functions as a neuropeptide in the central nervous system. Thus, ANP, BNP and CNP are most likely to function in concert with each other for maintaining the homeostatic balance of the body fluid volume and blood pressure.

The present identification of CNP in the rat gives a clue to solve the question as to whether or not a receptor specific for CNP is present. In this context, it should be

noted that natriuretic/diuretic and hypotensive activities of CNP are about 100 times less potent than those induced by ANP and BNP, while the rectum-relaxant effect of CNP is 3–4 times more potent than ANP [4]. Furthermore, CNP potently increases cGMP levels in cultured vascular smooth muscle cells, and the extent of the maximum elevation induced by CNP is 3.2 times higher than that by ANP [6]. Such a pharmacological dissociation of CNP from ANP and BNP, combined with regional differences in their expression, suggests a possible existence of a receptor specific for CNP.

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